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Diversity of environmental and clinical *Pseudomonas aeruginosa* isolates

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Abstract

Background and Aims

The bacterium *Pseudomonas aeruginosa* is a versatile environmental organism which is capable of persisting within a diverse range of habitats. For people with cystic fibrosis (CF), *P. aeruginosa* is the most common bacterial species isolated from respiratory secretions and infection is associated with poor health outcomes and decreased life expectancy.

Molecular-based epidemiological studies have shown that patients with CF can harbour indistinguishable genotypes (shared strains) of *P. aeruginosa* that have not been isolated from natural or commonly encountered environments sources. It is speculated that these shared strains are acquired by person-to-person transmission and have become highly niche-adapted to the CF airway. However, the specific mechanisms relating to the transmission, host adaptation and persistence of these strains remain uncertain.

By thoroughly characterising *P. aeruginosa* isolates from a defined cohort of patients this thesis aims to determine the prevalence and diversity of strains among adults with CF, and using phenotypic and genomic methods, to explore the key differences pertaining to adaptation between shared and unique strains of *P. aeruginosa* strains sourced from a range of ecological settings.

Methods

Longitudinal analysis of respiratory pathogens from adults with CF was undertaken to determine the prevalence of *P. aeruginosa* and other major CF pathogens. Univariate analysis was conducted to determine change in prevalence over time.

P. aeruginosa isolates at two time points from adults with CF underwent single nucleotide polymorphism (SNP) based genotyping to assess the incidence and prevalence of shared strains. Statistical analyses was undertaken to find associations between acquisition of shared strains and health outcomes.

Bacterial motility and adhesion was determined for clinical (CF, non-CF and animal) and environmental *P. aeruginosa* isolates. Each assay was performed under varied atmospheric conditions. Regression analysis was performed to assess an association between genotype and phenotype.

CF and environmental *P. aeruginosa* isolates were grown a dynamic flow cell system to allow for the development and visualisation of biofilm structures. Following incubation and staining, three dimensional images were captured and resulting data analysed using Comstat software. Mixed effects models were used to compare relevant biofilm parameters.

Bioinformatic analysis was performed on CF and environmental *P. aeruginosa* isolates. Genetic variants associated with phenotypic characteristics were identified.

Results

The prevalence of significant airway pathogens declined over the study period. For paediatric patients at the time of transition, this decline was correlated with an improvement in lung function. Interestingly, the prevalence of chronic *P. aeruginosa* infection remained stable.

While the proportion of patients harbouring the shared strain AUST-02 declined over time, there was a marked increase in those infected with the AUST-06 strain. In contrast, the prevalence of AUST-01 and other non-shared strains remained stable. Patients who acquired AUST-06 reported the largest decline in lung function over time and patients harbouring AUST-02 showed an increased risk of death or lung transplantation.

Environmental, animal and non-CF isolates displayed enhanced motility and adherence to a plastic surface in the microtitre assay; while those collected from CF patients, particularly shared strains displayed a reduction in these phenotypic characteristics. Incubation under anaerobic conditions further reduced phenotypes for all isolates.

Unlike results from the microtitre assay, all isolates assessed using the flow cell system demonstrated adherence to the glass substrate and the ability to form biofilms. Morphologically all isolates were similar, although CF isolates produced significantly thicker biofilms with a greater biomass when compared to the environmental isolates.

Bioinformatic analysis identified a range of genetic variants associated with the assessed phenotypic characteristics. Non-synonymous mutations in genes associated with recognised virulence genes, in particular those which regulate motility and biofilm formation, were identified in most strains.

Conclusions

This thesis demonstrated that *P. aeruginosa* is still the most common pathogen isolated from adults with CF; although the prevalence of this organism has declined in recent years. Furthermore, despite few changes in patient management there has been an overall shift in the epidemiology of shared strain infections within this patient cohort.

P. aeruginosa has developed a range of mechanisms in order to avoid attack by the host immune system and killing via antimicrobial substances. One of these mechanisms is growth within a protective biofilm structure. Although motility and adhesion, two traits required for biofilm development, were reduced amongst the CF isolates, they were still capable of forming a biofilm. Discordance of the bacterial adherence and flow cell assays provide evidence that the static microtitre assay may not be suitable for assessing biofilm forming capacity of *P. aeruginosa* isolates derived from the CF airway.

Finally, a bacterial GWAS approach identified genetic variants suitable for use as targets in future studies aimed at determining the genetic mechanisms of these phenotypic characteristics.

Overall, the results of this thesis demonstrate that adaptation and virulence of *P. aeruginosa* to the airways is multifactorial, complex and influences from the environment, host and the bacterium itself all affect its ability to infect and persist.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-reviewed papers

Syrmis, M. W., R. J. Moser, T. J. Kidd, P. Hunt, **K. A. Ramsay**, S. C. Bell, C. E. Wainwright, K. Grimwood, M. D. Nissen, T. P. Sloots and D. M. Whiley (2013). High-throughput single-nucleotide polymorphism-based typing of shared *Pseudomonas aeruginosa strains* in cystic fibrosis patients using the Sequenom iPLEX platform. *J Med Microbiol 62(Pt 5): 734-740*.

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Letters

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Publications included in this thesis

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Contributor	Statement of Contribution
Kay A. Ramsay	Conception and design (50%) Data collection (40%) Data analysis (40%) Writing (55%) Formatting and editing (25%)
Harpreet Sandhu	Data collection (15%) Formatting and editing (7.5%)
James B Geake	Data collection (15%) Formatting and editing (7.5%)
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Contributions by others to the thesis

All work contributing to this thesis, including research design, development, and execution of all experiments and the writing of each chapter was primarily my responsibility as a PhD candidate. Contributions to the manuscript generated from this work by co-authors are specifically listed in the preceding "Publications included in this thesis" section of this thesis. Consultation of experimental design and study concepts, editing and proof-reading of this thesis was performed by Dr Timothy Kidd, Associate Professor David Whiley, Associate Professor David Reid and Professor Scott Bell.

Statement of parts of the thesis submitted to qualify for the award of another degree

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Keywords

Pseudomonas aeruginosa, cystic fibrosis, environmental, biofilm, motility, adaptation, shared strains, phenotype

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Fields of Research (FoR) Classification

FoR code: 1108 Medical Microbiology	50%
FoR code: 0606 Physiology	25%
FoR code: 0601 Biochemistry and Cell Biology	25%

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List of Abbreviations

ºC	Degrees Celsius
%	Percentage
µm	Micrometre
3D	Three dimensional
3-oxo-C12-HSL	3-oxo-C12-homoserine lactone
ACFC	Adult CF Centre
ACPinCF	Australian Clonal Pseudomonas in CF Study
AGRF	Australian Genomic Research Facility
AHL	*N*-acyl-homoserine lactone
AnO_2/An	Anaerobic
ANOVA	Analysis of variance
ASL	Airway surface liquid
AUSLAB	Queensland pathology management system
AUST-01	AES-I, Melbourne Strain, Pulsotype 1
AUST-02	AES-II, Brisbane Strain, Pulsotype 2
AUST-06	Pulsotype 42
BAL	Bronchoalveolar lavage
BF	Biofilm formation
BG	BURST group
BG01	BURST group 01
BLAST	Basic local alignment search tool
BLAST	Basic Local Alignment Search Tool
BMI	Body mass index
BURST	Based upon related sequence types
c-di-GMP	cyclic diguanylate/Cyclic-di-GMP
CF	Cystic fibrosis
CFF	Cystic Fibrosis Foundation (North America)
CFRD	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming units
CI	Confidence interval
Clone C	Clone C strain
CLSM	Confocal laser scanning microscope
CO_2	Carbon Dioxide
COMSTAT	Computer program to analyse 3D biofilm images
del	Deletion
DLV	Double locus variant
DNA	Deoxyribonucleic acid
dup	Duplicate

EPS	Extracellular polymeric substances
ERIC-PCR	enterobacterial repetitive intergenic consensus PCR
FEV_1	Forced expiratory volume in one-second
FS	Frame-shift mutation
FVC	Forced vital capacity
G+C	Guanine-cytosine content
GATK	Genome Analysis Tool Kit
GFP	Green fluorescent protein
GNB	Gram negative bacilli
GWAS	Genome wide association studies
HREC	Human Research Ethics Committee
ICC	Intraclass correlation coefficients
ID	Internal diameter
IDC	Indwelling catheter
IFM	In-frame mutation
indel	insertion and deletions
ins	Insertion
IQR	Interquartile range
L	Litre
LB	Luria broth
LES	Liverpool epidemic strain
MA	Microaerophilic
MALDI-TOF	Matrix Assisted Laser Desorption Ionization-Time-of-Flight
MAN	Manchester epidemic strain
Mbp	Mega base pair(s)
mL	millilitre
MLST	Multilocus sequence type
mm	millimetre
mM	millimolar
MRSA	methicillin-resistant *Staphylococcus aureus*
MSSA	methicillin-sensitive Staphylococcus aureus
NF-GNB	Non-fermenting GNB
NI	Nucleotide identity
nm	nanometre
non-CF	Human clinical sources excluding cystic fibrosis
NSM	Non-synonymous mutation
NTM	Nontuberculous mycobacteria
O_2	Oxygen/Aerobic
OD	Optical Density
OP	Oropharyngeal
OR	Odds ratio

p.F508del	CFTR mutation showing a phenylalanine deletion at codon 508
Pa	*Pseudomonas aeruginosa*
PAO1	Widely characterised *P. aeruginosa* isolate
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PQS	Pseudomonas quinolone signal
PROVEAN	Protein variation effect analyser
QS	Quorum sensing
RBWH	Royal Brisbane and Women's Hospital
RCH	Royal Children's Hospital, Queensland
rpm	Revolutions per minute
SD	Standard deviation
SM	Synonymous mutation
SNP	Single nucleotide polymorphism
spp.	Species
ST	Sequence type
T4P	Type IV Pili
TPCH	The Prince Charles Hospital
TPCHF	The Prince Charles Hospital Foundation
UK	United Kingdom
uL	Microlitre
UQ	The University of Queensland
USA	United States of America
USM	Upstream mutation
UV	Ultraviolet
WGS	Whole genome sequence

Chapter 1: Review of Literature

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa can be found throughout natural and man-made environmental settings and is considered to be a medically important bacterium. Many of the traits which make *P. aeruginosa* so successful in adapting to new environmental challenges also make it capable of causing chronic infection in vulnerable people.

The earliest report describing the genus Pseudomonas came in the late 19th century from the German botanist Walther Migula. He visualised motile cells with spores resembling the nanoflagellate *Monas spp.*. Hence the name, Pseudomonas, was generated from the identification of this false or 'pseudo' *Monas spp.* nanoflagellate (Palleroni 2010).

The genus Pseudomonas belongs to the phylum Proteobacteria, and the family Pseudomonadaceae. The most clinically significant species of this genus is *P. aeruginosa.* This bacterium is a motile, non-spore forming; gram-negative bacillus (GNB) ranging in size from 0.5 to 3.0 μm. Colonial morphology can vary widely, from the more common non-mucoid, flat spreading colonies with rough edges, to small colony and mucoid forms. *P. aeruginosa* is capable of surviving in a range of environmental conditions including increased temperature and reduced oxygen (Gilligan *et al.* 2006; Kidd *et al.* 2011). The inability to ferment glucose places this bacterium into a broader group referred to as non-fermenting GNBs. Other bacteria in this group include Achromobacter species, *Stenotrophomonas maltophilia* and *Burkholderia cepacia* complex (Brenner *et al.* 2005).

P. aeruginosa requires iron for growth and metabolic functions such as respiration and synthesis of deoxyribonucleic acid (DNA) (Saha *et al.* 2013). In a low iron environment, *P. aeruginosa* is able to sequester free iron using iron chelating compounds called siderophores. The water soluble siderophores produced by *P. aeruginosa* are pyoverdine and pyochelin (Saha *et al.* 2013). Under ultraviolet (UV) light, the expression of pyoverdine is indicated by a yellow-green fluorescence. Additional pigments produced by *P. aeruginosa* are pyocyanin, pyomelanin and pyorubin, which result in the blue, brown and red hues seen in bacterial colonies (Kodaka *et al.* 2003; Ogunnariwo and Hamilton-Miller 1975).

1.1.1 Environmental influences effecting the acquisition of *Pseudomonas aeruginosa*

Given the minimum nutritional requirements needed to support growth, and its capacity to survive under a range of environmental and physical conditions, *P. aeruginosa* is widely distributed

throughout the environment. Recent studies indicate that in household settings, *P. aeruginosa* is most commonly found in moist areas, particularly drains (Purdy-Gibson *et al.* 2015; Remold *et al.* 2011), but also other water-rich locations including swimming pools, as well as non-household environments such as river water, and it can also be isolated from vegetables and antiseptic solutions (Kidd *et al.* 2012; Mena and Gerba 2009; Ogbulie *et al.* 2008; Pirnay *et al.* 2005; Remold *et al.* 2011; Zubko and Zubko 2013). *P. aeruginosa* has also been isolated from soil where it behaves as a saprophyte. Furthermore, many species of Pseudomonas, including *P. aeruginosa* play a role in bioremediation and pollution control (Silby *et al.* 2011).

1.1.2 Clinical significance of *Pseudomonas aeruginosa*

Owing to its large genome encompassing many regulatory genes, *P. aeruginosa* is able to readily adapt to new and changing environments (Silby *et al.* 2011). It also possesses a number of mechanisms which contribute to enhanced virulence and antimicrobial resistance. *P. aeruginosa* has high levels of intrinsic resistance and the capacity to acquire resistance to most antibiotic classes (Llanes *et al.* 2013). *P. aeruginosa* can also develop and grow within a sessile community structure which affords protection from antibiotics, host defence mechanisms, desiccation, UV light and disinfectants (Hogardt and Heesemann 2013).

As a result of these enhanced tolerability and virulence traits *P. aeruginosa* is described as an opportunistic pathogen capable of causing acute and chronic infection in humans and animals (Kidd *et al.* 2012; Silby *et al.* 2011). Exogenous acquisition, primarily from exposure to environmental sources, is considered the most common route of infection. Community acquired infection include keratitis, skin infection, and otitis media and externa. Nosocomial sources are also important contributors to infection (Driscoll *et al.* 2007).

1.1.2.1 *Pseudomonas aeruginosa* infection in animals

In animals, *P. aeruginosa* has been reported as the most commonly isolated non-lactose fermenting GNB in samples cultured from a range of wild and domestic species (Kidd *et al.* 2012; Mathewson and Simpson 1982). It has been identified as the aetiological agent in a variety of infections (Allen *et al.* 2011; Diamond *et al.* 1979; Doerning *et al.* 1993; Freeman *et al.* 2009; Hariharan *et al.* 2006; Kidd *et al.* 2011; Lin and Petersen-Jones 2008; Lin and Petersen-Jones 2007; Momotani *et al.* 1995; Petersen *et al.* 2002; Salomonsen *et al.* 2013; Tiago *et al.* 2012; Wada *et al.* 2010; Westgate *et al.* 2011).

1.1.2.2 *Pseudomonas aeruginosa* infection in humans

P. aeruginosa is an opportunistic pathogen capable of causing acute or chronic infection and is rarely found colonising healthy persons. Indeed, large studies examining the microbiota of the skin, gastrointestinal and respiratory tracts of healthy individuals have shown that species from the phyla Firmicutes, Actinobacteria, non-Pseudomonas Proteobacteria and Bacteroidetes predominate, whereas Pseudomonas in particular is associated with relatively low bacterial biomass of potentially intermittent and environmental origin (Human Microbiome Project Consortium 2012; Koboziev *et al.* 2014; Sanford and Gallo 2013). This was highlighted in a recent study of healthy subjects by Charlson and colleagues suggesting that the presence of Pseudomonaceae in control samples was indicative of environmental contamination (Charlson *et al.* 2011).

In humans, *P. aeruginosa* can cause nosocomial or community acquired infection similar to those observed in animals (Ott *et al.* 2013). It has been isolated from contact lens fluid causing keratitis and corneal ulcers, otitis media and externa, wound and skin infection, urinary tract infection, pneumonia and septicaemia (Bodey *et al.* 1983; Chalmers *et al.* 2014; Deb and Ray 2012; Hattemer *et al.* 2013; Hoddenbach *et al.* 2014; James *et al.* 2008; Kiem and Schentag 2013; Obritsch *et al.* 2004; Schaefer and Baugh 2012; Scheetz *et al.* 2009; Yildiz *et al.* 2012; Zilberberg and Shorr 2013). In the healthy host, respiratory infections are rapidly cleared by both the innate and acquired immune systems. Functioning mucociliary clearance and a range of immunological factors prevents the infection persisting. However, *P. aeruginosa* can cause chronic infections in people with diabetes, severe burns and the genetic disorder, cystic fibrosis (CF) (Buivydas *et al.* 2013; Fekih Hassen *et al.* 2014; Gibson *et al.* 2003; Shanmugam *et al.* 2013).

1.1.2.2.1 *Pseudomonas aeruginosa* infection in people with cystic fibrosis

In people with CF, *P. aeruginosa* is the most common bacterial species isolated from respiratory secretions. Acquisition of this bacterium is associated with accelerated pulmonary decline, reduced quality of life and decreased life expectancy (Emerson *et al.* 2002; Konstan *et al.* 2007; Sanders *et al.* 2014). While the precise mechanisms of *P. aeruginosa* acquisition in people with CF are poorly understood, a growing body of evidence suggests that the local environment, geographic location and the climate in which people are residing may play a role. Seasonal effects, ambient temperature and the proximity to water, all impact on exposure to *P. aeruginosa* and subsequent infection in people with CF (Ramsay *et al.* 2016). Acquisition of *P. aeruginosa* occurs during childhood, although the highest prevalence is reported in adults where more than 60% of patients are infected (Cystic Fibrosis Australia 2016; Cystic Fibrosis Foundation 2015; Cystic Fibrosis Trust 2016).

As a result of the impaired function of the CF transmembrane conductance regulator (CFTR), the lungs of people with CF provide a heterogeneous environment. Within the airways there are varied oxygen gradients, a range of different microorganisms, thick sticky mucus and circulating and local antimicrobial substances. To survive and persist within this challenging environment, *P. aeruginosa* must adapt through a number of evolutionary processes (Winstanley *et al.* 2016).

From initial infecting isolates which possesses a range of virulence factors, *P. aeruginosa* may undergo a number of genetic mutations and biological changes which result in the downregulation of many virulence mechanisms (Winstanley *et al.* 2016). Once *P. aeruginosa* infection is established in the CF airways, eradication is difficult to achieve and often leads to chronic infection.

1.2 Cystic Fibrosis

1.2.1 Disease mechanisms in cystic fibrosis

CF is the most common life-threatening autosomal recessive genetic disease in Caucasians and is characterised by mutations in the CFTR gene. The first CFTR mutation was discovered in 1989, and since then more than 2000 mutations have been described (Cystic Fibrosis Mutation Database ; Kerem *et al.* 1989; Riordan *et al.* 1989). The CFTR gene encodes a chloride channel on the surface of epithelial cells where it is expressed. Based on the analysis of more than 160 disease-causing mutations, the genetic abnormalities can be divided into six classes (Boyle and De Boeck 2013). Severe sweat chloride and pancreatic phenotypes are seen in patients with two Class 1 to 3 mutations, and milder phenotypes are seen in patients with at least one Class 4 to 6 mutations (Bell *et al.* 2015).

The most common CFTR mutation (referred to as p.F508del) is a deletion of the phenylalanine at position 508 of the CFTR protein. In Australia, 51.3% of patients with CF are homozygous for p.F508del, and a further 41.8% are heterozygous for this mutation (Cystic Fibrosis Australia 2016). In Australia, CF is observed in approximately 1 in ~3000 live births, with 1 in 25 of the population carriers of a CFTR mutation (Bell *et al.* 2011; Massie and Delatycki 2010; Massie *et al.* 2005).

CF is a multi-system disease and the major organs which are affected include the lungs and upper respiratory tract, the gastrointestinal tract (including the hepatobiliary system) and the reproductive tract in males. The most severe and life limiting complication is chronic bronchial sepsis with associated inflammation, resulting in the structural airway changes of bronchiectasis (O'Sullivan and Freedman 2009; Ratjen *et al.* 2015).

In healthy persons, the CFTR protein is responsible for maintaining the airway surface liquid (ASL) layer by controlling the movement of chloride, sodium and water within cells, thus ensuring the

functioning of cilia, which enables mucociliary clearance and protects the airways. When a mutation occurs to impair the functioning of the CFTR, the ASL is compromised, with altered composition and volume. Reduced mucociliary clearance, decreased ion and water transfer, and increased sodium absorption result in viscous mucus being trapped within the airways. This triggers a cycle of bacterial infection and inflammation, leading to cellular damage, bronchiectasis, respiratory failure and premature death (O'Sullivan and Freedman 2009; Ratjen *et al.* 2015).

Changes in the dynamics of bacterial colonisation of the respiratory tract are seen during a patient's lifetime. In childhood, the most commonly isolated bacteria are *Staphylococcus aureus* and *Haemophilus influenzae*, but by adolescence and early adulthood these bacteria have often been replaced by a dominant growth of *P. aeruginosa* (Chmiel *et al.* 2014; Cystic Fibrosis Australia 2015; Gibson *et al.* 2003; Lipuma 2010). Eradication regimes implemented by paediatric CF centres aim to eradicate initial P. aeruginosa infection, thereby limiting the early onset of chronic infection (Doring *et al.* 2012; Langton Hewer and Smyth 2014; Mayer-Hamblett *et al.* 2015; Ratjen *et al.* 2010; Treggiari *et al.* 2007; Wainwright *et al.* 2011). Through the reduction of chronic infection and successful infection control policies, the prevalence of *P. aeruginosa* has been decreasing over recent years. This is particularly evident in the adult population (Bell *et al.* 2011; Cystic Fibrosis Australia 2015; Cystic Fibrosis Canada 2015; Cystic Fibrosis Foundation 2015; Cystic Fibrosis Trust 2016). Due to treatment developments and centre-based care from a multi-disciplinary medical team, the health and median survival for patients with CF has dramatically improved over the last two decades. As a result, there are now a greater proportion of older patients with CF who have better lung function and nutrition compared to patients from previous generations, although respiratory failure still remains the leading cause of morbidity and mortality (Buzzetti *et al.* 2009; Reid *et al.* 2011).

1.2.2 Microbiology of the cystic fibrosis airways

1.2.2.1 Microbial prevalence

Infection of the CF airway is classically associated with an ensemble of microbial species including *Staphylococcus aureus*, *P. aeruginosa*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, *Achromobacter spp.*, *Burkholderia cepacia* complex, and *Aspergillus spp*. (Lipuma 2010). Lung microbiota composition evolves with age (Figure 1.1). Initial colonisers of the airways, often associated with early childhood, include methicillin sensitive *S. aureus* and *H. influenzae*. Intermittent infection caused by susceptible strains of *P. aeruginosa* can also be isolated during early life and these strains can be successfully eradicated via antibiotic therapy. Over time and often correlated with increasing age and prolonged antibiotic exposure, chronic infection with resistant *P. aeruginosa* strains becomes the classic scenario (Proesmans *et al.* 2006; Ratjen *et al.* 2007).

In Australian patients, the prevalence of other highly antibiotic resistant bacteria, such as methicillin resistant *S. aureus* increases with patients' age, although rates are low when compared to international CF data registries. It is surmised that acquisition occurs due to frequent exposure to the general hospital population, explaining why rates of infection are greater in adult patients (Chmiel *et al.* 2014; Cystic Fibrosis Australia 2016; Cystic Fibrosis Foundation 2015; Gibson *et al.* 2003; Lipuma 2010; Surette 2014). Infection with other less common pathogens including a range of non-fermenting gram negative bacilli (e.g. *S. maltophilia*, *Achromobacter spp.*, *B. cepacia* complex, *Inquilinus limosus*, *Pandoraea spp*. and *Ralstonia spp.*), fungal and non-tuberculous mycobacteria (NTM) species have also emerged in the CF population in recent years (Bar-On *et al.* 2015; Parkins and Floto 2015; Qvist *et al.* 2015; Ramsay *et al.* 2016; Surette 2014).

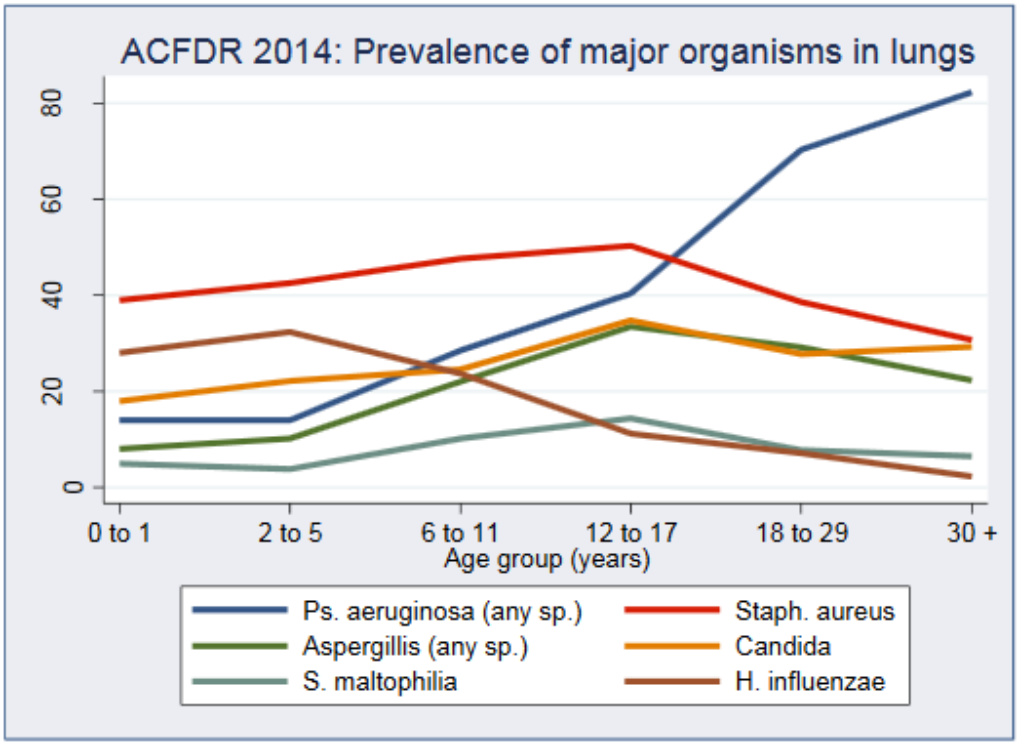


Figure 1.1 The change in prevalence of the major airway pathogens isolated from people with cystic fibrosis over a lifetime.

Permission obtained (Cystic Fibrosis Australia 2016)

1.2.2.2 Microbiome of the cystic fibrosis airway

The microbiome of the CF airways is complex and dynamic and can be influenced through numerous host, environmental and treatment factors. It has been succinctly described as "diverse, frequently

polymicrobial and constantly evolving" (Zemanick and Hoffman 2016). However, until recently the traditional view of respiratory infection focused on the dominant bacterial pathogens which could be readily identified using culture-dependent techniques (Burns and Rolain 2014). Rapid advances in the field of next-generation metagenomic sequencing has demonstrated that conventional culture-dependent identification has vastly underestimated the diversity and abundance of the flora present in the airways of people with CF (Caverly *et al.* 2015). For example, anaerobic bacteria previously thought not to be present in the CF airways are now frequently encountered using culture-independent microbiome techniques (Sherrard *et al.* 2016).

Increased life expectancy due to improvements in clinical interventions has been the success story of CF multi-disciplinary medical care in recent years, but this benefit has coupled with a number of detrimental health impacts. With increased patient longevity have come more intensive antibiotic treatments, increased exposure to bacteria and a longer life with very advanced lung disease. The cumulative effect of antibiotics, lung damage and bacterial exposure has seen the emergence of bacterial species not previously isolated, many of which are highly antibiotic resistant. In addition, numerous more genera have now been identified via the development of culture-independent techniques, but their relevance remains uncertain (Zemanick and Hoffman 2016).

Microbiome studies conducted to date have shown four key findings in regards to the airway microbiota in people with CF. Firstly, higher degrees of bacterial community diversity and species richness have been observed in young patients, those with mild CF lung disease phenotypes and those who have received fewer antibiotic treatments (Coburn *et al.* 2015; Cox *et al.* 2010; Cystic Fibrosis Mutation Database ; Zhao *et al.* 2012). The most abundant species identified in these patients include those considered to be 'normal oropharyngeal flora', anaerobes and pathogens traditionally associated with early CF lung disease, such as Haemophilus and Staphylococcus (Cox *et al.* 2010; Rogers *et al.* 2015; Surette 2014). Secondly, as patients age there appears to be an overall loss of bacterial diversity (fewer species) although the overall number of bacteria increases (abundance) (Coburn *et al.* 2015; Cox *et al.* 2010; Stokell *et al.* 2015; Zhao *et al.* 2012). Thirdly, decreased diversity is associated with the emergence of dominant genera that most frequently occur in patients with severe pulmonary disease (i.e. reduced lung function). Dominant genera frequently include Pseudomonas and Burkholderia (Coburn *et al.* 2015; Cox *et al.* 2010; Zemanick and Hoffman 2016; Zhao *et al.* 2012). Finally, despite clinical interventions and antibiotic treatment there is little overall variation in the microbiome of individual patients. Furthermore the clinical status, either stable or during pulmonary exacerbation, of the patients does not affect the stability of the dominant pathogens present (Carmody *et al.* 2013; Fodor *et al.* 2012; Stressmann *et al.* 2012).

Other investigations have demonstrated that changes in the microbiome distribution can occur during childhood, implying that disease progression may take place earlier than previously thought (Boutin *et al.* 2015). Furthermore, a correlation between CFTR severity, loss of airway diversity and increasing prevalence of the genus, Pseudomonadaceae has been also shown (Cox *et al.* 2010).

1.2.2.3 Upper airway involvement in infection

It has been postulated that the upper airways, particularly the paranasal sinus region, acts as a reservoir for *P. aeruginosa*. Similar to the changes which occur in the lower airways as a result of impairment to the CFTR functioning, the upper airways of people with CF are similarly affected. Symptoms noted in the sinuses include inflammation, thickening of the airway mucus and reduced mucociliary clearance (Chang 2014).

As a result of these physiological changes, the sinus area is capable of harbouring bacteria that may migrate to the lungs, resulting in respiratory infections. The microbial diversity which is seen in the lungs also occurs in the upper airway of people with CF (Caverly *et al.* 2015; Digoy *et al.* 2012; Dosanjh *et al.* 2000; Mainz *et al.* 2009; Mak and Henig 2001; Muhlebach *et al.* 2006). Currently, the exact role and significance the upper airway plays in initial and recurrent infection of *P. aeruginosa* is unclear. It is also uncertain whether failure of eradication therapy is associated with ongoing colonisation of the sinuses. Interestingly, sinus surgery in conjunction with antibiotic therapy has been shown to reduce the prevalence of *P. aeruginosa* in the lower airway for some patients (Alanin *et al.* 2016). In a case study from Linnane and colleagues, in which they reviewed a single patient, it was demonstrated that sinus infection can persist over an extended period of time without resulting in lung function decline or evidence of lower respiratory tract infection (Linnane *et al.* 2015). Work from Denmark also shows that in both paediatric and adult cohorts, genotypically indistinguishable *P. aeruginosa* strains can be isolated simultaneously from the sinus and lungs of patients with intermittent and chronic infection (Hansen *et al.* 2011; Johansen *et al.* 2012). Similar findings were obtained from a large patient cohort assessing the prevalence and relatedness of *P. aeruginosa* (Mainz *et al.* 2009). Results from a paediatric cohort study using bronchoalveolar lavage (BAL) and oropharyngeal (OP) swab samples were less clear. While initial eradication therapy was successful in treating the lower respiratory tract infection, retention of the same strains within the oropharynx, following treatment, was frequently encountered. These results suggest that a reservoir, refractory to antibiotic treatment, exists within the upper airway, and that this has the potential to re-infect the lower airways (Kidd *et al.* 2015). These results demonstrate a new paradigm in the definition of

successfully treating *P. aeruginosa* infection and how eradication is defined clinically and microbiologically.

1.2.3 Epidemiology of *Pseudomonas aeruginosa* in cystic fibrosis

1.2.3.1 Infection status

In general, the evolution of airway disease caused by *P. aeruginosa* colonisation and infection in individuals with CF follows a well recognised timeline. Initial colonisation often occurs during childhood and is commonly caused by the acquisition of environmental strains which are susceptible to antibiotics (Burns *et al.* 2001; Kidd *et al.* 2015; Ranganathan *et al.* 2013). Current standards of care recommend eradication therapy may be initiated at the time of first isolation to prevent an infection ensuing (Mogayzel *et al.* 2014). However, following a period of intermittently isolating *P. aeruginosa* from the airways, an intermittent infection, also caused by antibiotic susceptible strains, often develops (Mayer-Hamblett *et al.* 2015; Mogayzel *et al.* 2014; Ratjen *et al.* 2010; Wainwright *et al.* 2011). The goal of eradication therapy is to either prevent or delay the development of a persistent infection (Mogayzel *et al.* 2014). Despite the widespread use of antimicrobial therapy and successful initial eradication, ultimately many patients will go onto develop a chronic infection. Prolonged exposure to antibiotics and adaptation of the bacteria to the airways are two factors which influence the development of a chronic infection.

Chronic infection is defined as a persistent growth which is not cleared despite antibiotic treatment. Currently there is limited consensus on how to define a chronic infection within a clinical setting (Pressler *et al.* 2011). For the purpose of this thesis, the following terms have been defined in accordance with the definitions proposed by Lee and colleagues (Lee JCF 2003). Based on quarterly sputum sample collection, chronic infection occurs when 50% or more of sputum samples are culture positive for *P. aeruginosa* within the previous 12 months and intermittent infection occurs when 50% or fewer sputum samples are culture positive for *P. aeruginosa* or when less than four samples are collected during the previous 12-month period. A range of clinical studies have demonstrated that following the development of a chronic infection, patients have poorer health outcomes. Compared to patients with no *P. aeruginosa*, initial colonisation or an intermittent infection, chronically infected patients are older, are more likely to have mucoid colony phenotypes, have higher specific *P. aeruginosa* serum antibody titres, have a greater requirement for antibiotics and lower lung function measurements and body mass index (BMI) (Ballmann *et al.* 1998; Lee *et al.* 2003; Pressler *et al.* 2006; Pressler *et al.* 2009; Proesmans *et al.* 2006; Ratjen *et al.* 2007). Therefore, correctly categorising patients based on their infection status is clinically paramount for determining long-term health outcomes, treatment regimens and cohort segregation. The two measures routinely used to

determine chronicity are the isolation of the bacteria using traditional culture techniques and the identification of specific *P. aeruginosa* antibody levels within serum samples.

Studies which define chronicity based on *P. aeruginosa* culture vary according to: i) the minimum number of positive sputum or upper airway samples required to be collected within a given timeframe, ii) whether consecutive positive culture results are required, and iii) the overall time in which the sampling is to occur (Ballmann *et al.* 1998; Demko *et al.* 1995; Hoiby 1974; Lee *et al.* 2003). For those studies which describe chronicity using serum antibodies alone or in conjunction with airway samples, a number of different *P. aeruginosa* specific antibodies have been suggested, but again the actual definition varies. Some studies have used a defined range to differentiate chronic from no Pseudomonas and intermediate infections, whereas other determine that a 'rise' in antibody precipitins from normal is indicative of a positive result (Hoiby *et al.* 1977; Pressler *et al.* 2006; Pressler *et al.* 2009; Proesmans *et al.* 2006; Ratjen *et al.* 2007).

Both these existing methods used to describe a clinical infection of *P. aeruginosa* have limitations. Firstly, for patients who are non-productive, such as young children and those with mild lung disease, relying on culture results alone may underestimate the rates of infection and miss initial colonisation events. Furthermore, it is widely recognised that the airways and lungs of people with CF are regionally heterogeneous and samples such as sputum may underestimate infection status. While the use of serological testing may confirm the presence of *P. aeruginosa* prior to culture results, especially for those non-productive patients, it is difficult to confirm an active chronic infection from a colonisation event. The misdiagnosis of patients as either colonised or infected will impact on whether eradication or maintenance therapy is administered. Finally, these current studies categorise patients at a predetermined time point, with no assessment of previous infection status recorded. Again, due to sampling bias and the limitations with airway sample collection, this approach may also underestimate infection rates. Therefore, a comprehensive definition of chronic infection needs may need to include longitudinally collected historical culture results, current culture results, current *P. aeruginosa* specific antibody levels and antibiotic treatment regimens. However, due to the discordant definitions currently existing in the literature, this is difficult to achieve.

While it is generally recognised that eradication of persistent infection through intensive antibiotic therapy is not possible, suppressive antibiotic treatment has proven very helpful in maintaining lung function and the health of patients over the long term.

1.2.3.2 Acquisition and colonisation of *Pseudomonas aeruginosa*

As *P. aeruginosa* is widely distributed within the environment, it was initially believed that the primary source of acquisition for most patients with CF was the environment (Burns *et al.* 2001). Recent data generated using modern genotyping techniques provides additional support for this hypothesis with two independent infant cohort studies showing a predominance of commonly encountered environmental genotypes during early *P. aeruginosa* infection (Kidd *et al.* 2015; Ranganathan *et al.* 2013). Likewise, most of the earlier epidemiological studies demonstrate that person-to-person transmission rarely occurred, with most patients harbouring their own unique strains (Grothues *et al.* 1988; Romling *et al.* 1994). Nevertheless, sharing of indistinguishable strains was also described between siblings and in instances of intimate or close hospital contact, presumably through common source exposure or potentially through person-to-person spread (Govan 2000; Grothues *et al.* 1988; Schmid *et al.* 2008; Silbert *et al.* 2001; Spencker *et al.* 2000; Tümmler *et al.* 1991). With advances in molecular genotyping techniques, it has now been shown that unrelated patients can often harbour genotypically indistinguishable (or shared) strains (Aaron *et al.* 2010; Armstrong *et al.* 2003; Cheng *et al.* 1996; Kidd *et al.* 2013; Scott and Pitt 2004).

A variety of different shared strains have now been isolated from patients throughout Europe, North America and Australia, some of which are associated with increased hospitalisation and worse clinical outcomes (Fothergill *et al.* 2012; Kidd *et al.* 2013). Despite extensive sampling of home and natural environmental settings, no environmental reservoirs have been found for these shares strains of *P. aeruginosa* (Jones *et al.* 2003; Kidd *et al.* 2013). Alternative routes of transmission have been explored, with airborne transmission of cough aerosols recently suggested as playing a potential role in cross-infection (Knibbs *et al.* 2014; Wainwright *et al.* 2009).

Infection control guidelines and segregation policies have now been implemented worldwide to limit the potential for cross infection (Conway *et al.* 2014; Saiman *et al.* 2014). Australian CF centres segregate patients on the basis of *P. aeruginosa* infection status during outpatient clinic visits and hospital admission (Cheng *et al.* 1996; Edenborough *et al.* 2004; Kidd *et al.* 2015; Pedersen *et al.* 1986). In some centres patients are also placed into cohorts according to their *P. aeruginosa* genotype (Ashish *et al.* 2013; Griffiths *et al.* 2005; Kidd *et al.* 2015). Government Health Departments now recognise the importance in CF of single rooms and providing private facilities while patients are hospitalised. In addition, other initiatives have been implemented to reduce the spread of *P. aeruginosa*. For example, the North American CF Foundation (CFF) has changed their Infection Control Guidelines to prevent more than one patient with CF attending an organised CFF event (Jain and Kazmierczak 2014; Shepherd *et al.* 2014).

1.2.4 Identification and Genotyping of *Pseudomonas aeruginosa*

1.2.4.1 Respiratory sampling

OP swabs, sputum and BAL samples are the most commonly collected respiratory samples assessed for microbiological composition and antimicrobial susceptibility from individuals with CF.

In young children incapable of expectorating sputum, OP swabs are collected as a surrogate for lower airway samples. A number of studies have demonstrated that, whilst not truly representation of the lower airway microbiology, this sample collection type is sufficient for routine surveillance (Armstrong *et al.* 1996; Kidd *et al.* 2015; Rosenfeld *et al.* 1999; Wainwright *et al.* 2011). Compared with BAL, OP swabs are non-invasive and simple to collect and culture. The CFF Pulmonary Clinical Practice Guidelines Committee has recommended that OP swabs be used to determine *P. aeruginosa* infection rather than BAL samples in non-productive patients (Mogayzel *et al.* 2014).

Sputum samples are the most common samples collected to assess the composition of airway microorganisms from people with CF. The majority of adults with CF are productive and are able to easily produce sputum for testing. While the collection of sputum samples is non-invasive and well tolerated by most patients, there are limitations to this type of sampling. The lungs are a heterogeneous environment and sputum samples may not provide a representative sample of the whole airway. Furthermore, sputum samples are better suited to assess the presence or absence of microorganisms rather than the specific quantification of them. As such, there are inherent limitations in using sputum to determine colony forming units, specifically owing to the difficulties encountered when trying to prepare serial dilutions of purulent and mucoid samples.

Samples obtained through BAL procedures are specific to the area of the lung from which they are collected. Additionally, as a result of the collection process, these samples are less viscous and have a lower mucus content, compared to sputum samples, therefore making them well suited to quantify the microorganisms present. However, BAL samples are costly to obtain and are invasive, requiring the patient to undergo general anaesthesia. Whilst BAL samples are not routinely collected, especially from adult patients, they are most commonly collected from children unable to provide a sputum sample (Burns and Rolain 2014).

1.2.4.2 Laboratory identification of *Pseudomonas aeruginosa*

Traditional culture and microscopy methods are most commonly used to isolate and identify *P. aeruginosa* from a range of sample types. *P. aeruginosa* grows readily on a range of non-selective agar including nutrient agar and broth, blood agar and MacConkey agar. Typically, the bacteria are

incubated in aerobic conditions at 37 °C, although *P. aeruginosa* is capable of growth at temperatures ranging from 4 °C to 45 °C (Burns and Rolain 2014; Gilligan *et al.* 2006).

Routine identification of *P. aeruginosa* usually follows a simple algorithm, including identification of small, slightly curved gram negative rods, typical colonial morphology, oxidase positive, growth at 42 °C and susceptibility to colistin sulfate (Kidd *et al.* 2011). Furthermore various pigments produced by the bacterium can also assist in visual identification.

Diagnostic laboratories utilise a range of commercial identification systems to confirm identification, these include API 20NE (bioMérieux) and VITEK®2 (bioMérieux). In recent years the addition of an automated microbiological identification system, using MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time-of-Flight) technology into diagnostic laboratories has allowed for rapid, cheap and accurate identification of bacterial pathogens (Burns and Rolain 2014). Most studies agree that this identification platform produces reliable results for the majority of bacterial samples undergoing testing, although a few reports have demonstrated limitations in the database which can result in either misidentification or non-identification (AbdulWahab *et al.* 2015; Alby *et al.* 2013; Homem de Mello de Souza *et al.* 2014).

Isolates from patients with CF may pose some difficulties in identification due to: i) the changes in metabolic and phenotypic characteristics during adaptation within the lung; ii) the polymicrobial nature of the clinical specimens; and iii) the isolation of non-fermenting GNBs (NF-GNB) with similar phenotypic profiles (e.g. *Burkholderia sp*, *Achromobacter spp*., *S. maltophilia* and *Inquilinus limosus*) that can be incorrectly identified as *P. aeruginosa* (Burns and Rolain 2014; Cystic Fibrosis Australia 2015; Kidd *et al.* 2009; Speert *et al.* 1990).

With the development of respiratory microbiome analyses we now know that the diversity of microorganisms we have traditionally isolated using the aforementioned routine culture techniques describes only a small component of the bacterial and fungal population found in the CF airways (Caverly *et al.* 2015; Zemanick and Hoffman 2016). As microbiome analyses are costly, time consuming and require particular expertise to analyse they are currently not feasible for routine assessment on a regular basis. Therefore for the foreseeable future, traditional culture-based techniques will continue to provide physicians with diagnostic information on which to base their clinical decisions.

1.2.4.3 Molecular identification of *Pseudomonas aeruginosa*

To overcome difficulties encountered when phenotypically and biochemically identifying *P. aeruginosa*, molecular-based identification methods have been developed, including a range of polymerase chain reaction (PCR) assays targeting *P. aeruginosa*-specific genes (Anuj *et al.* 2009; da Silva Filho *et al.* 1999; De Vos *et al.* 1997; Jaffe *et al.* 2001; Khan and Cerniglia 1994; Lavenir *et al.* 2007; Qin *et al.* 2003). Increased sensitivity and specificity of *P. aeruginosa* PCR identification is also best achieved by using an assay with two different targets simultaneously (Anuj *et al.* 2009; Qin *et al.* 2003).

1.2.4.4 Genotyping of *Pseudomonas aeruginosa*

In light of the potential for cross-infection and poorer health outcomes associated with acquisition of shared *P. aeruginosa*, strains accurate diagnosis of these strains is vital. Techniques employed to determine strain relatedness must have high discriminatory power and typeability, be reproducible between laboratories, and be both time and cost effective. Various techniques have been developed focusing on different molecular methodologies. Two examples of fingerprint-based methods used to determine relatedness are pulsed-field gel electrophoresis (PFGE) which exploits low frequency restriction endonuclease recognition sites and the PCR-based method enterobacterial repetitive intergenic consensus (ERIC)-PCR which focuses on repetitive sequences throughout the genome (Renders *et al.* 1996; Syrmis *et al.* 2004; Tenover *et al.* 1995).

To overcome limitations associated with the processing, analysis and interpretation of fingerprint based methods, categorical-based genotyping techniques have also been developed in recent years. The ArrayTube assay is a rapid and reproducible method used to type both the core and accessory genome of *P. aeruginosa*. DNA obtained from extension PCR using *P. aeruginosa* specific oligonucleotides is hybridised onto a commercially developed chip and, following conjugation, a binary code is obtained which is used to categorically genotype the bacteria (Wiehlmann *et al.* 2007).

Multilocus sequence typing (MLST) characterises strains (or sequence types [STs]) on the basis of allelic changes among seven conserved housekeeping genes (Curran *et al.* 2004; Jolley and Maiden 2010; *Pseudomonas aeruginosa* PubMLST Database 2012). Highly informative MLST-based single nucleotide polymorphisms (SNP) have also been utilised in real-time PCR and Sequenom iPLEX-based strain typing assays, which allow for rapid, accurate and cheap testing to determine genetic relatedness (Anuj *et al.* 2011; Syrmis *et al.* 2013). A protocol based on twenty informative SNPs has been developed which will differentiate most clinically important STs individually or group them into closely related sub-groups (Syrmis *et al.* 2014). This methodology is specifically relevant for Australian patients with CF as it categorically differentiates the two major clonal strains, AUST-01,

AUST-02, from other strains. One limitation of MLST, and therefore SNP typing is that these methods only assess the diversity within the core genome.

1.2.5 Shared strains of *Pseudomonas aeruginosa*

Sharing of *P. aeruginosa* strains was first described when ceftazidime-resistant *P. aeruginosa* isolates from patients with CF attending a clinic in Liverpool, United Kingdom were observed. Molecular surveillance confirmed that the isolates were indistinguishable and this strain became known as the Liverpool Epidemic Strain (LES). Follow-up environmental studies failed to identify common sources for this genotype within the hospital setting (Al-Aloul *et al.* 2004; Cheng *et al.* 1996). The LES strain has subsequently been detected elsewhere in the United Kingdom (UK) and on other continents, including a substantial number of patients from Ontario, Canada (Aaron *et al.* 2010; Kidd *et al.* 2013; Scott and Pitt 2004). Since then, numerous studies have shown that patients can share the same strain of *P. aeruginosa*.

Different shared strains have been isolated in Australia, Germany, England, Wales, Canada, The Netherlands and Belgium (Aaron *et al.* 2010; Armstrong *et al.* 2002; Ballmann *et al.* 1998; Cheng *et al.* 1996; de Vrankrijker *et al.* 2011; Dinesh *et al.* 2003; Jones *et al.* 2001; Kidd *et al.* 2013; O'Carroll *et al.* 2004; Romling *et al.* 1994; Scott and Pitt 2004; Speert *et al.* 2002; Van Daele *et al.* 2005; van Mansfeld *et al.* 2009). Some shared strains have been associated with poorer clinical outcomes, increased treatment requirements and premature death (Aaron *et al.* 2010; Al-Aloul *et al.* 2004; Armstrong *et al.* 2003; Armstrong *et al.* 2002; Bradbury *et al.* 2008; Cheng *et al.* 1996; Kidd *et al.* 2013; O'Carroll *et al.* 2004). For many shared strains, an environmental reservoir has yet to be found, despite extensive sampling of communal areas within the CF clinics and hospitals, patients' homes and the local environment (Armstrong *et al.* 2003; Bradbury *et al.* 2009; Cheng *et al.* 1996; Jones *et al.* 2001; Kidd *et al.* 2012; McCallum *et al.* 2001). The absence of many of these strains in the environment is indicative of host specificity and indirectly supports widespread cross-infection and/or niche specialisation. Indeed, the LES and Manchester epidemic strain (MAN) have been isolated from areas adjacent to infected patients, but no persistent environmental reservoir has been found (Panagea *et al.* 2005).

One possible route of acquisition is airborne transmission, with studies showing that when patients cough, they expel viable *P. aeruginosa* in aerosol droplets within the respirable size range (Knibbs *et al.* 2014; Wainwright *et al.* 2009). *P. aeruginosa* can be readily isolated 4 meters from a patient during a coughing episode and up to 45 minutes after the cessation of coughing (Knibbs *et al.* 2014).

In contrast, several other *P. aeruginosa* strains shared by patients with CF do frequently occur in other ecological settings. For example, the shared CF strain from Germany, Clone C, has been isolated from a diverse range of environmental sources in Germany and many other settings around the world (Kidd *et al.* 2012; Scott and Pitt 2004; Wiehlmann *et al.* 2007). Likewise, several other shared strains (e.g. ST-155, ST-179) occurring in relatively small clusters of CF patients are frequently encountered in other clinical presentations and natural environmental settings (Kidd *et al.* 2012; *Pseudomonas aeruginosa* PubMLST Database 2012).

1.2.5.1 Australian shared strains of *Pseudomonas aeruginosa*

In the early 2000s, the first account of an Australian shared CF *P. aeruginosa* strain was reported in a paediatric clinic in Melbourne (Armstrong *et al.* 2003; Armstrong *et al.* 2002; Griffiths *et al.* 2005). This arose serendipitously when genotyping of *P. aeruginosa* isolates from children participating in a prospective infant cohort identified the same strain among five children who had died unexpectedly (Nixon *et al.* 2001). Subsequent screening of all patients with *P. aeruginosa* in the CF centre revealed that 55% of children shared the same strain (Armstrong *et al.* 2002). A few years later the same strain, designated "Pulsotype 1" (also known as the "Australian Epidemic Strain-I" or the "Melbourne strain") was described in unrelated patients in Sydney and Brisbane (Anthony *et al.* 2002; Armstrong *et al.* 2003; O'Carroll *et al.* 2004). In a 2004 prevalence study, O'Carroll and colleagues described a new shared strain (Pulsotype 2) in patients from Brisbane, and reported a prevalence of 59% within their cohort. When compared to patients with unique strains, those with Pulsotype 2 were younger and had poorer lung function (O'Carroll *et al.* 2004).

In a national cross-sectional study published in 2013, Kidd and colleagues, showed that 62% of Australian CF patients with *P. aeruginosa* harboured a shared strain, with unique strains isolated from only 38% of patients. Pulsotypes 1 and 2, (renamed AUST-01 and AUST-02) were isolated in 22% and 18% of patients, respectively. AUST-01 and AUST-02 infections were associated with increased treatment requirements and outpatient clinic visits (Kidd *et al.* 2013). Another important observation was that AUST-01 commonly appeared in patients residing in south eastern Australia, whereas AUST-02 was found mainly in patients residing in Brisbane and Perth (Kidd *et al.* 2013). Given the large geographical distances between CF clinics and the substantial number of participants, this study also provides evidence that exposure to common environmental sources was not responsible for the acquisition of AUST-01 and AUST-02. Despite conducting a large epidemiological study, Kidd and colleagues did not identify AUST-01 or AUST-02 strains from any *P. aeruginosa* isolates collected from the natural environment, non-CF patient infections or animal samples (Kidd *et al.* 2012). While

this was not an extensive national study testing a wide variety ecological settings, these data suggest that these strains have evolved fairly recently and are now adapted to the CF airways.

1.2.5.2 Unique strains of *Pseudomonas aeruginosa*

Unique strains are distinct strains found in individual patients. Acquisition of unique strains of *P. aeruginosa*, commonly thought to be sourced from the environment, still occurs. In a recent study by Kidd and colleagues, unique strains were more commonly identified among children when compared to adults (Kidd *et al.* 2013). Furthermore, the majority of *P. aeruginosa* strains associated with infections in very young children with CF are also commonly encountered in the environment (Kidd *et al.* 2012). Such strains show genetic variability when analysed by PCR strain typing methods (Kidd *et al.* 2011), but often belong to five distinct sequence types (ST-155, ST-179, ST-253, ST-266, and ST-381) which are common across various ecological niches in south east Queensland (animal and non-CF acute infections, environmental sampling and CF sputum collection), as well as in other national and international settings (Garcia-Castillo *et al.* 2011; Johnson *et al.* 2007; Khan *et al.* 2008; Kidd *et al.* 2015; Kidd *et al.* 2012; Maatallah *et al.* 2011; Nemec *et al.* 2010; *Pseudomonas aeruginosa* PubMLST Database 2012; Ranganathan *et al.* 2013; van Mansfeld *et al.* 2009; Waine *et al.* 2009).

1.2.6 Genome of *Pseudomonas aeruginosa*

In 2000 Stover and colleagues published the whole genome sequence of *P. aeruginosa* strain type PAO1, and since then numerous strains have had their whole genomes sequenced (Pseudomonas Genome DB ; Stover *et al.* 2000; Winsor *et al.* 2016; Winsor *et al.* 2011). PAO1 was originally isolated from a wound infection in 1955 (Holloway 1955), and since then has been widely characterised. In many studies, PAO1 is used as a baseline or comparator strain to which the wild-type isolates are compared (Carter *et al.* 2010; Jeukens *et al.* 2014; Kukavica-Ibrulj *et al.* 2008; O'May *et al.* 2006; Yang *et al.* 2011).

P. aeruginosa has a very large and complex genome (6.3 Mbp; G+C content 66.6%) consisting of two components, the core and accessory genomes. The highly regulated and conserved core genome makes up approximately 90% of the whole genome found in all strains. The functionality of many genes located within the core genome has been described, and they include genes responsible for respiration, antibiotic resistance, and biofilm formation (Dettman *et al.* 2013). The remainder of the genome is made up of the smaller accessory genome and comprises a range of genetic material which varies between strains. The accessory genome is the major driver behind the evolution of this organism, in particular the gain or loss of genetic material through horizontal gene transfer. In

addition, deletions, mutations and rearrangements also occur within the accessory genes. It has been noted that the accessory genome encodes various genes that enable increased survival and niche adaptation within specific strains (Dettman *et al.* 2013; Kung *et al.* 2010; Stewart *et al.* 2014).

1.2.7 Population structure of *Pseudomonas aeruginosa*

P. aeruginosa has been described as having a non-clonal epidemic population structure characterised by numerous singleton strains and a small number of abundant genotypes, and large BURST groups (BGs) consisting of three or more STs (i.e. clonal complexes) (Kidd *et al.* 2012; Maatallah *et al.* 2011; Pirnay *et al.* 2009). The largest BG (termed BG01) comprises numerous strains collected from a range of ecological settings, including the shared CF strains AUST-02 and the LES. It is also widely recognised that the majority of *P. aeruginosa* strains are globally distributed and there appears to be little correlation between ecological setting and genotype. This implies that most strains, irrespective of geographical or ecological origin, are capable of establishing infection in the susceptible host. Indeed, commonly encountered strains (e.g. ST-155, ST-179, ST-253, ST-266 and ST-381) have been isolated from patients with CF and numerous other non-CF infections (Kidd *et al.* 2015; Kidd *et al.* 2012; Ranganathan *et al.* 2013; Scott and Pitt 2004; van Mansfeld *et al.* 2009; Wiehlmann *et al.* 2007). In contrast, some of the most frequently encountered shared CF strains (AUST-01, AUST-02, AUST-06 from Australia, LES from the UK and the Dutch strains ST-406 and 497) have rarely been isolated outside of the CF lung. These data provide evidence of host specificity and indirectly support the potential for widespread cross-infection and niche specialisation of these strains.

1.3 Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway

A range of mechanisms now referred to as pathoadaptive traits, have been identified to characterise the change which occurs when a bacterium causing an acute, virulent, early infection develops into one causing a persistent, chronic infection. This transformation is the result of a number of evolutionary changes which occur as *P. aeruginosa* adapts to the CF airway. These changes include a range of phenotypic adaptions, genomic mutations and metabolic variations. Loss of motility and type III secretion, enhancement of mucoidy, increased antibiotic resistance, development of small colony variants, changes to the lipopolysaccharide, O-antigen and pyocyanin expression, auxotropy, defects to bacterial communication and hypermutability are all traits which comprise the pathoadaptive process (Cullen and McClean 2015; Hauser *et al.* 2011; Hogardt and Heesemann 2010; Sousa and Pereira 2014). Many of these changes are interlinked, thus enhancing the bacterium's capacity to withstand eradication by the host immune system and by antibiotic therapy within this unique environmental condition (Folkesson *et al.* 2012).

Early infecting strains of *P. aeruginosa* are frequently described as motile, non-mucoid, primarily acquired from environmental sources, having increased susceptibility to antibiotics and existing in a free-living or planktonic state (Luzar *et al.* 1985; Smith *et al.* 2006), whereas isolates obtained from chronic infection demonstrate reduced virulence and increased antimicrobial resistance. Chronic infection isolates are also often mucoid in appearance, have lower expression of pyocyanin, are non-motile and frequently possess mutations in associated virulence genes. Furthermore, bacteria isolated from chronic infections can be found living in community formations and cellular aggregates (Harmer *et al.* 2013; Hogardt and Heesemann 2010; Manos *et al.* 2013).

While many studies demonstrate this cascade of adaptive mechanisms during transition from initial to chronic infection, not all work is confirmatory. In a study conducted by Mayer-Hamblett and colleagues, they demonstrated that a proportion of phenotypic changes typically recognised in chronic infection were identified in initial infecting isolates from paediatric patients prior to the onset of conventionally diagnosed chronic infection (Mayer-Hamblett *et al.* 2014). Explanations for these results include i) these changes are intrinsic to the bacteria rather than a product of the environmental conditions in which they exist (Hauser *et al.* 2011), ii) adaption within the sinuses, prior to migration to the lower airway, has already taken place (Hansen *et al.* 2011; Johansen *et al.* 2012; Manos *et al.* 2013; Markussen *et al.* 2014) and iii) adaptation has already occurred in one CF patient prior to acquisition by another as a result of patient-to-patient transmission.

Additionally, large variations of these phenotypic characteristics have been noted within and between patients, in isolates from a range of ecological and clinical niches, and, interestingly, also from genetically identical strains (Clark *et al.* 2015; Cullen *et al.* 2015; Darch *et al.* 2015; Harmer *et al.* 2013; Mayer-Hamblett *et al.* 2014). These studies clearly show that *P. aeruginosa* has a high degree of phenotypic heterogeneity (Cullen *et al.* 2015).

Recently, whole genome sequencing, expression studies and transcriptome analyses have enhanced our existing knowledge of adaptive mechanisms. Overall, these studies demonstrate that, although the genome of *P. aeruginosa* is well conserved showing modest levels of genetic variability, both non-synonymous mutations and recombination events do play an important role in phenotypic diversification (Darch *et al.* 2015; Grosso-Becerra *et al.* 2014; Harmer *et al.* 2013; Markussen *et al.* 2014; Marvig *et al.* 2015). Such studies have identified a range of virulence genes associated with the phenotypic characteristics displayed by *P. aeruginosa* during infection (Marvig *et al.* 2015; Marvig *et al.* 2015).

Currently the exact mechanisms which drive adaptation and diversification are not fully understood, although antibiotic pressure, the heterogeneity of the airways, and the natural versatility of *P. aeruginosa* all play a significant role in this process (Dettman *et al.* 2013; Markussen *et al.* 2014; Sousa and Pereira 2014).

1.3.1 Respiration

To aid survival *P. aeruginosa* has adapted to a variety of fluctuating environmental conditions, including the ability to grow under diverse atmospheric conditions (Alvarez-Ortega and Harwood 2007; Comolli and Donohue 2002; O'May *et al.* 2006; Yoon *et al.* 2002). Although traditionally referred to as an aerobic bacterium, due to the ability survival and grow under reduced oxygen concentrations, it is now often described as a facultative anaerobe. *P. aeruginosa* can be exposed to a large fluctuation of oxygen levels depending on setting and surround environmental conditions. Many of the specific physiological conditions of the CF airways, such as increased mucus production, increased absorption of salts and hypoxic areas, as well as specific traits such as the capacity to grown in reduced oxygen environments, all contribute to the varied respiration pathways which *P. aeruginosa* can utilise (Hogardt and Heesemann 2013; Hoiby *et al.* 2010; Worlitzsch *et al.* 2002).

The preferred mechanism of *P. aeruginosa* for respiration is via the aerobic pathway using oxygen as the final electron receptor as this form of respiration is the most efficient in terms of replication, producing the highest energy yield and enhanced rates of growth (Alvarez-Ortega and Harwood 2007; Hogardt and Heesemann 2013; Schobert and Jahn 2010). However, a number of studies have described the growth characterisitics of *P. aeruginosa* under different oxygen concentrations. This work has shown that as oxygen concentrations decrease there is a corresponding increase in the doubling time of growth. Furthermore, data confirms that while aerobic growth is the most efficient, growth under microaerophilic conditions is preferable to growth anaerobically. In addition, under anaerobic conditions, it has been demonstrated that the expression of many genes and virulence factors are impaired. Motility is reduced, quorum sensing (QS) is down regulated and biofilm formation and maturation occurs more slowly (Alvarez-Ortega and Harwood 2007; Hogardt and Heesemann 2013; Lee *et al.* 2011; O'May *et al.* 2006; Schobert and Jahn 2010; Waite and Curtis 2009; Wu *et al.* 2005; Yoon *et al.* 2002).

Many enzymes, compounds, pathways and genes have been shown to play an important role in respiration, biofilm formation, dispersal and motility. These include, the oxidases enzymes, in which mutations have been shown to impede microaerophilic respiration and biofilm production, ii) nitrate or nitrite, compounds used as an alternative final electron receptor, to facilitate growth under

anaerobic conditions, known as denitrification, and iii) regulators such as Anr, Pseudomonas quinolone signal (PQS) and RhlR, which are integral for anaerobic growth, denitrification and quorum sensing, biofilm maturation and motility. Mutations in these genes have been shown to impair many of these functions (Alvarez-Ortega and Harwood 2007; Barraud *et al.* 2009; Hassett *et al.* 2002; Hogardt and Heesemann 2013; Kolpen *et al.* 2014; Schobert and Jahn 2010; Yoon *et al.* 2002).

Methods assessing motility and surface adhesion lend themselves to testing under various atmospheric conditions. As *P. aeruginosa* requires the presence of an alternative electron acceptor for growth in anaerobic conditions, the addition of potassium nitrate to the medium helps support growth under reduced oxygenation (O'May *et al.* 2006; Yoon *et al.* 2011; Yoon *et al.* 2002). However, despite the fact that these studies are not technically difficult, little is actually known about the impact of microaerophilic conditions on *P. aeruginosa* motility and biofilm development, and currently there are limited data pertaining to the phenotypic behaviours of clinically important strains grown in anaerobic conditions.

1.3.2 Quorum Sensing

Bacterial communication or QS is the term given to the regulation of bacterial behaviour in response to fluctuations in cell population density. This form of cell-to-cell communication is based on the generation and release of signalling molecules called autoinducers. QS is often referred to as being 'density-dependent' as a critical density of bacterial cells is required for autoinducer levels reach a threshold stimulatory concentration, bind to a transcriptional activator protein, resulting in the expression of the target gene (De Kievit and Iglewski 1999; De Kievit and Iglewski 2000; Eberl and Tummler 2004; Hoiby *et al.* 2011; Miller and Bassler 2001). This coordinated communication is associated with a range of important physiological activities. Studies have demonstrated that mutations in genes involved in QS pathways affect biofilm formation, adhesion, motility and virulence (Bjarnsholt *et al.* 2010; Davies *et al.* 1998; Heydorn *et al.* 2002; Pearson *et al.* 2000).

Within *P. aeruginosa* two quorum sensing systems have been described, the las and rhl systems. These systems do not act independently of each other and there is a hierarchy in the signalling cascade. In both of these systems a synthase and activator protein are present. LasI, the synthase protein of the las system, produces the autoinducer 3-oxo-C12-homoserine lactone (3-oxo-C12-HSL) that binds to the transcriptional activator protein (LasR) at a threshold concentration. The resulting LasR-3-oxo-C12-HSL complex is then able to bind to promotor regions upstream of genes encoding for a range of virulence factors (such as elastase, exotoxin A and alkaline protease) and also regulates the rhl system. Through the induction of the rhl transcriptional activator protein, RhlR, binding with

C4-homoserine lactone (C4-HSL) occurs, leading to the synthesis of the autoinducer synthase protein (RhlI). This complex, Rh1R-C4-HSL, can also activate the transcription of additional virulence factors (such as rhamnolipid, alkaline protease, elastase, pyocyanin and cyanide) and can activate genes under the control of LasR. These systems do not act independently of each other and there is a hierarchy in the signaling cascade with the las system regulating the AHL proteins of the rhl system (Eberl and Tummler 2004). In addition to the aforementioned AHL autoinducer signaling molecules, a third autoinducer, 2-heptyl-3-hydroxy-4-quinolone, is produced by *P. aeruginosa*. This autoinducer, referred to as PQS, facilitates interaction between the las & rhl systems and has a role in the expression of their proteins (Bjarnsholt *et al.* 2010; De Kievit and Iglewski 2000; Eberl and Tummler 2004; Miller and Bassler 2001).

Specific environmental conditions and host immune factors have been associated altering *P. aeruginosa* QS response. Both phosphate and iron are essential requirements for survival and energy production. In times of limitation, *P. aeruginosa* has been shown to increase QS and in-turn virulence factor expression. Under anaerobic conditions, transcriptional regulators in conjunction with the QS systems allow for continued bacterial growth and virulence expression. Finally, examples of the interaction between *P. aeruginosa* and host immune factors have demonstrated an increase in the QS activity in the presence of immune cells (Lee and Zhang 2015). By understanding the mechanisms and conditions which promote bacterial QS, this may provide the basis for future therapeutic targets aimed at limiting cellular communication and bacterial infection.

Loss of QS functionality has been described as being greater in isolates from chronically infected patients with CF compared to those from patients with intermittent infections (Bjarnsholt *et al.* 2010). Furthermore, it has been shown that the level of signalling molecules reduces as infection duration increases (Jiricny *et al.* 2014). Studies exploring QS gene expression demonstrated decreased expression from shared strains compared to non-shared strains from CF patients. This phenotype was more evident within the biofilm producing cells when compared to planktonic cells, suggesting QS is no longer necessary in established biofilm infection (Manos *et al.* 2009; Manos *et al.* 2008). Additionally, strain type and host environment may also be influencing expression and phenotype (Salunkhe *et al.* 2005). Finally, it has also been shown that the expression of these molecules is suppressed under anaerobic conditions, further impacting the cells' ability to communicate when growing in oxygen-limited environments, such as the CF airway and within biofilms (Lee *et al.* 2011; Yoon *et al.* 2002) (Figure 1.2).

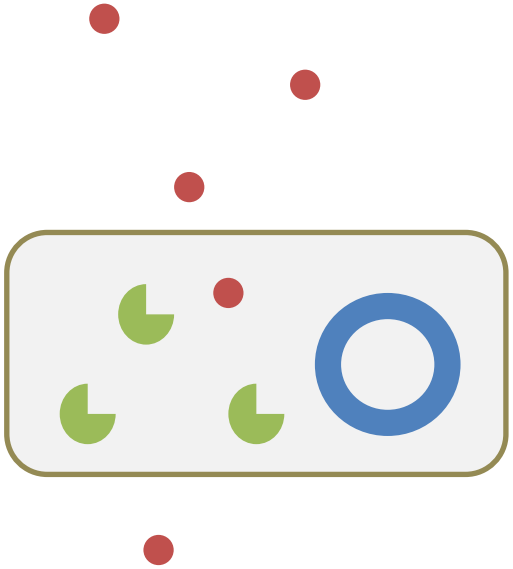
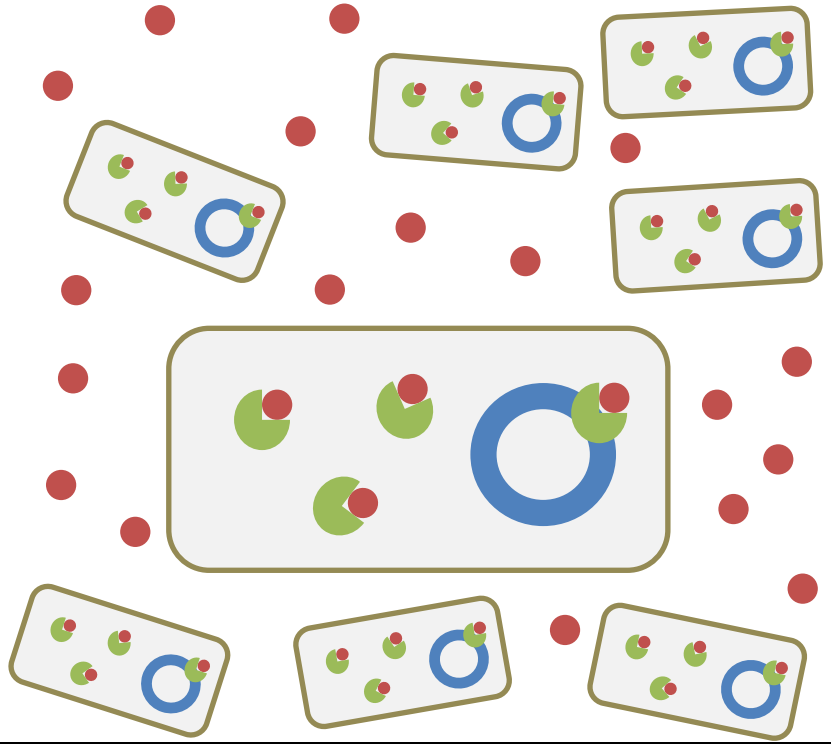
LOW CELL DENSITY	HIGH CELL DENSITY
	
Planktonic cells Low levels of autoinducers Individual behaviour No quorum sensing No phenotypic changes	Bacterial community High levels of autoinducers Collective behaviour Quorum sensing Gene expression causing phenotypic changes

Figure 1.2 The effect of cell density and autoinducer concentration on gene expression.

1.3.3 Motility

P. aeruginosa has the ability to utilise a range of motility mechanisms to colonise abiotic and biotic surfaces, and to form biofilms (Davey and O'Toole G 2000; Harshey 2003). *P. aeruginosa* utilises the cell surface organelles, flagella and pili, for motility. The three predominant forms of motility are termed 'swimming', 'swarming' and 'twitching' (Harshey 2003; Mattick 2002; Partridge and Harshey 2013).

Swimming through liquid medium and swarming across a solid surface requires propulsion and torque provided by terminal polar flagella (Harshey 2003; Murray and Kazmierczak 2006; Partridge and Harshey 2013). The flagellum is comprised of a basal body and a motor situated within the cell membrane, a rod which extends through the peptidoglycan and outer layers of the cell, to which the hook and filament are attached. The tail like filament of the flagellum structure consists of the protein flagellin (Guttenplan and Kearns 2013; Harshey 2003).

Regulation of flagellum development, rotation and cellular movement requires a complex series of interactions involving flagellum gene expression, chemotaxis and flagellum movement (Guttenplan and Kearns 2013; Murray and Kazmierczak 2006). Swimming and swarming actions are driven by a rotating flagellum which provides *P. aeruginosa* with forward propulsion, though there are fundamental differences distinguishing the two motility functions. Swimming motility is unicellular movement within liquid propelled by single polar flagellum on the bacterium. Anticlockwise flagellum rotation results in forward propulsion, whereas clockwise revolutions move the bacteria in a tumbling motion (Harshey 2003; Partridge and Harshey 2013). Swimmer cells use chemical signals to alter the direction of movement in response to chemotaxis (Figure 1.3A) (Harshey 2003).

Swarmer cells must overcome three main challenges to allow movement across a surface, including dehydration, friction and water tension. Consequently, the bacterium synthesises and secretes osmoprotectant molecules to draw water to the surface and increase hydration. The production of biosurfactants (e.g. rhamnolipids) assists with reducing friction and overcoming the surface tension between water molecules. Several other mechanisms include the formation of swarmer cell raft aggregations that facilitate movement in a coordinated fashion, and increasing the number of flagella from one to two, which in turn increases the force used to provide movement (Figure 1.3B) (Partridge and Harshey 2013).

Twitching motility involves the movement of cell surface appendages called pili. For colonisation and infection of host cells to occur, pathogenic bacteria must recognise receptors on host cells in order

to attach to them. In addition to motility, pili are involved in other cellular functions including DNA transfer, bacteriophage infection, conjugation, cell aggregation, biofilm formation and host cell invasion (Harshey 2003; Mattick 2002; Proft and Baker 2009). Pili protrude through the cell membrane at both poles and are the major cell surface organelles involved in surface adhesion. In *P. aeruginosa*, specific pili, Type IV pili (T4P), are synthesised within the cell and this process involves a range of proteins (PilA, PilC, PilB, PilQ) for both assembly and rapid disassembly, leading to twitching movement (Mattick 2002; Proft and Baker 2009). Bacteria engaged in twitching motility move in an ever expanding motion outwards (Figure 1.3C). This action results in small clusters of cells which join together to form a lattice-like framework of cells, with the leading edge actively involved in migration, attachment and biofilm formation. It is widely recognised that a transition occurs when bacteria switch from a motile free living organism to a non-motile organism growing within a biofilm community. One important signalling molecule which is involved in the transition from motile to sessile forms is Cyclic-di-GMP (c-di-GMP). High levels of this signalling molecule are known to impact upon motility and increase the formation of biofilms, whereas the degradation of this molecule favours biofilm dispersal and a return to a planktonic and motile lifestyle (Hogardt and Heesemann 2013).

Several studies have shown that motility and adhesion play important roles in triggering host immune responses, biofilm formation and the development of chronic infection (Alm and Mattick 1995; Amiel *et al.* 2010; Klausen *et al.* 2003; Yang *et al.* 2011). Furthermore, these studies demonstrate that the specific genetic mutations and the environmental conditions that the strains are exposed to can affect an organism's capacity to establish infection.

The most commonly used method of visualising the motility of *P. aeruginosa* is the inoculation of bacterial suspensions into media of differing viscosities. To demonstrate swimming motility, the bacterial suspension is inoculated into a semi-viscous medium. For swarming activity, the bacterial suspension is inoculated onto the surface of a semisolid medium with the organisms using flagella to propel themselves in an outward motion. Motility driven by the action of pili can be demonstrated using a semisolid agar and inoculating the isolate onto the plastic/agar interface at the base of a petri dish. The pili will attach to the plastic surface and the growth from twitching motility can then be visualised (Alm and Mattick 1995; Darzins 1993; Murray *et al.* 2010; O'May *et al.* 2006; Rashid and Kornberg 2000; Yoon *et al.* 2002).

Although the current literature discussing the motility of *P. aeruginosa* isolated from patients with CF reports varied findings depending on the particular strains studied, the strong consensus is that

motility is important during the initial infective process. As *P. aeruginosa* undergoes the transition from an acute to chronic infection, the level of motility reduces. However due to isolate selection, patient cohort and specific mechanisms inherent to the bacteria, studies have demonstrated discordant findings when demonstrating an association between motility and eradication success. An Australian based study showed that isolates which are susceptible to eradication are more motile than those strains that are able to persist (chronic infection) within the lungs (Harmer *et al.* 2013; Manos *et al.* 2013), whereas a European study showed there was a tread to greater motility in persistent isolates compared to those which were successfully eradicated (Tramper-Stranders *et al.* 2012). These results highlight the complexity of determining and reporting phenotypic characteristics.

Several studies have examined the motility characteristics of shared strains isolated from patients with CF. However, owing to the relatively small numbers of isolates analysed, comparisons across the existing literature are difficult, and inconsistencies between studies are apparent. Hare and colleagues have described similar motility results between AUST-01 and other laboratory strains, whereas Harmer and colleagues reported that AUST-01 had reduced motility during both early and late infections, when compared to unique strains. Finally, Manos and colleagues reported that planktonic AUST-01 displayed greater motility compared with unique strains. Additionally, it has been demonstrated that AUST-01 isolates in the biofilm stage can upregulate the flagellin-encoding gene *flaA*, consistent with the retention of motility throughout its entire life cycle (Hare *et al.* 2012; Harmer *et al.* 2013; Manos *et al.* 2008). These discordant results may be secondary to i) selective pressures that the specific bacterial strains studied were under at the time of collection from the lungs, ii) the large diversity seen within *P. aeruginosa* genome or iii) methodological variations within the testing protocols. Expression of *pilA* and retention of twitching motility has also been confirmed amongst a small number of AUST-02 strains (Manos *et al.* 2009). Studies focusing on the LES describe various motility patterns depending on the particular isolate studied (Carter *et al.* 2010; Fothergill *et al.* 2012; Jeukens *et al.* 2014; Kukavica-Ibrulj *et al.* 2008; Salunkhe *et al.* 2005). Finally, motility studies utilising a broad isolate selection, including shared strains, demonstrate that isolates obtained from patients with CF have decreased motility compared to other clinical and environmental isolates (Head and Yu 2004; O'May *et al.* 2006). Environmental conditions such as oxygen tension may also impact on motility, given the fact that it is an energy dependent process, but limited work has been undertaken in the field although reduced motility capabilities of *P. aeruginosa* under anaerobic conditions have been described in a small number of strains isolated from patients with CF (O'May *et al.* 2006). Further phenotypic analysis in parallel with genomic studies on a large well characterised collection of bacterial strains will help elucidate the mechanisms which control motility.

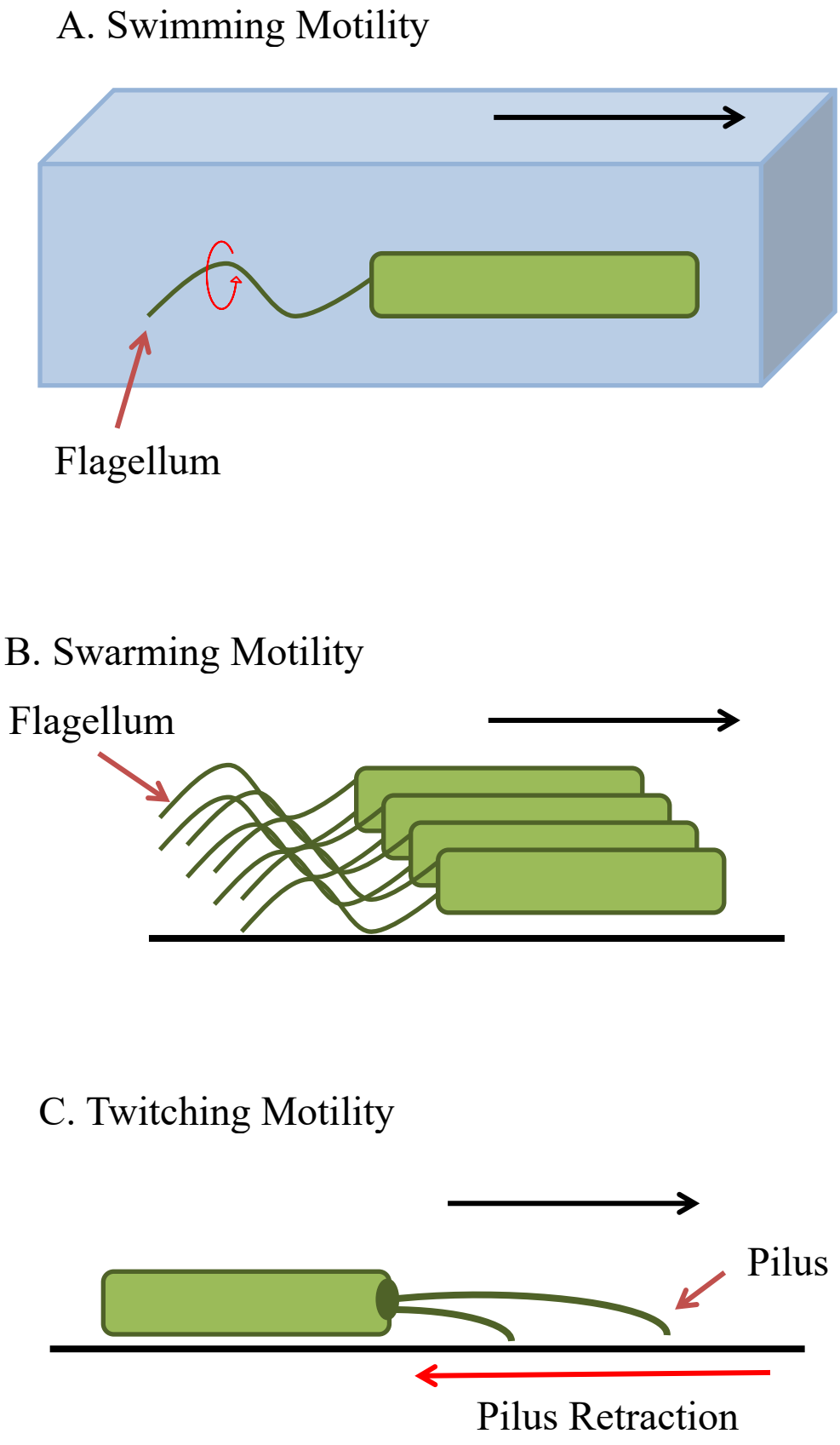


Figure 1.3 Depiction of the three types of bacterial motility which *Pseudomonas aeruginosa* can utilise during adaptation.

1.3.4 Biofilm Formation

Bacterial biofilms are complex communities consisting of microorganisms encased in a protective extracellular matrix (Costerton *et al.* 1995; Costerton *et al.* 1999; Hall-Stoodley *et al.* 2004). Biofilms can be either attached to biotic or abiotic surfaces (termed "Growth Mode 1") (Harshey 2003), or form free-floating unattached cellular aggregates (termed "Growth Mode 2") (Hassett *et al.* 2010; Su and Hassett 2012). The complex process of *P. aeruginosa* biofilm formation comprises five developmental stages, which typically occur under favourable environmental conditions involving numerous genes and regulatory pathways (Figure 1.4) (Harshey 2003).

Stage 1: Planktonic cells possessing cell surface appendages necessary for motility (T4P and flagella) attach to a surface and form a monolayer of cells. At this stage, surface attachment/aggregation is still very loose and the developing biofilm can be easily disrupted.

Stage 2: There is a continuous influx of motile cells to the site of biofilm initiation, which then adhere and begin to express extra-polymeric substances (EPS) resulting in irreversible surface attachment and microcolony formation.

Stage 3: Cellular proliferation and the loss of motility organelles mark the beginning of the maturation process.

Stage 4: Biofilm maturation and thickening is now complete. The initial flat mass of cells transforms into a three dimensional structure which can often resemble the stem and cap of a mushroom. An oxygen gradient forms throughout the structure, resulting in limited oxygenation at the base.

Stage 5: This final stage results in the dispersal of planktonic cells back into the environment. These "dispersal" cells are often motile, possess increased virulence and have the ability to seed onto surfaces and form new biofilms. Structurally, the cap of the biofilm collapses, allowing the planktonic cells to swim out and resulting in a hollow core consisting of persister cells. The process of dispersal can be triggered by environmental stimuli or a physiological event (Costerton *et al.* 1999; Hall-Stoodley *et al.* 2004; Hassett *et al.* 2002).

Another key development during biofilm maturation is the formation of water channels which facilitate the transfer and/or removal of nutrients, water and waste by-products throughout the structure. However, due to the heterogeneous nature of these channels, an uneven distribution of water and nutrients occurs resulting in differentiation of cellular function (Hall-Stoodley *et al.* 2004; Hogardt and Heesemann 2013). The cellular composition of a biofilm is divided into three broad groups: i) those bacterial cells which make up the basic structure of the biofilm; ii) cells which are expelled from the biofilm (dispersers); and iii) cells that remain (persisters) (Costerton *et al.* 1999; Hall-Stoodley *et al.* 2004). One of the defining features of the biofilm is the secretion of an extracellular matrix by the bacteria, thereby forming a protective coating or physical barrier,

comprising polysaccharides, extracellular DNA and proteins. EPS also provide structural support and aid in irreversible surface attachment. Importantly, this matrix plays an essential role in QS by facilitating the interactions of signalling molecules.

Unlike planktonic cells in the metabolically active exponential growth phase, cells within a biofilm maintain a relatively low level of metabolic activity. Coupled with the physical protection afforded by the biofilm matrix, subdued metabolic activity provides a degree of protection from bactericidal and bacteriostatic antibiotic activity.

Similar to results generated from motility assays, biofilm formation also appears to be strain specific. Studies focusing on the LES have demonstrated a range of biofilm characteristics amongst the isolates studied (Fothergill *et al.* 2012; Jeukens *et al.* 2014; Salunkhe *et al.* 2005; Winstanley *et al.* 2009). In addition, a range of results have been generated from unique strains possessing the same genetic background (Head and Yu 2004). Shared strains produce thicker and larger biofilms compared to unique strains, while isolates from chronically infected patients demonstrate enhanced biofilm forming capabilities compared to those cultured from initial infection (Harmer *et al.* 2013; Manos *et al.* 2008). However, when CF isolates have been compared to isolates cultured from other human clinical presentations, they display thinner and smaller biofilms which take longer to develop (O'May *et al.* 2006). A small number of environmental isolates have been tested under the same experimental biofilm-forming conditions as CF and non-CF clinical isolates. Although there is considerable variation amongst all the isolates, there is no distinguishing biofilm phenotypic feature that differentiates isolates according to origin. It has also been demonstrated that biofilm production does not change when tested at either 30 °C or 37 °C (Head and Yu 2004). Studies correlating motility capabilities with biofilm formation have provided discordant findings. Head and colleagues and Kukavica-Ibrulj and colleagues found no correlation between motility and biofilm formation, while O'May and colleagues and Jeukens and colleagues observed that non-motile isolates were also poor biofilm producers (Head and Yu 2004; Jeukens *et al.* 2014; Kukavica-Ibrulj *et al.* 2008; O'May *et al.* 2006). Although all of the aforementioned studies used the microtitre plate based assay (or a similar variation), inconsistencies within reported results may be due to variations in the specific methodology used. Results may therefore vary according to incubation protocols and the exact abiotic surface used.

Early work on seeding dispersal comparing isogenic mutants, a mucoid CF strain and PAO1, concluded that although all strains developed biofilms at very similar rates, biofilm thickness, hollowing and the ability to undergo seeding dispersal differed between strains. A QS mutant was

unable to disperse cells, leading to the conclusion that the ability to sense cell density and nutrients is required to initiate the release of cells (Purevdorj-Gage *et al.* 2005). Additionally, it has been shown that a mucoid isolate from a patient with CF could not initiate hollowing or seeding dispersal. Taken together, Purevdorj-Gage and colleagues speculated that seeding dispersal may be specific to environmental strains, while the transmission of clinical strains may occur independently of this mechanism (Purevdorj-Gage *et al.* 2005). More recently, analysis of a larger number of clinical CF strains has shown that biofilms produced by these isolates do undergo cellular dispersal. Kirov and colleagues found that, although isolates from chronically infected patients developed less adherent microcolonies than PAO1, and developed them at a slower rate, they still displayed similar seeding dispersal events. This active dispersal of motile cells back into the environment is thought to enhance genetic diversity and survival of *P. aeruginosa*. Effluent collected from flow cell experiments demonstrates that a range of phenotypically distinct isolates originating from a single colony morphotype as the original inoculum are released following seeding dispersal (Kirov *et al.* 2007).

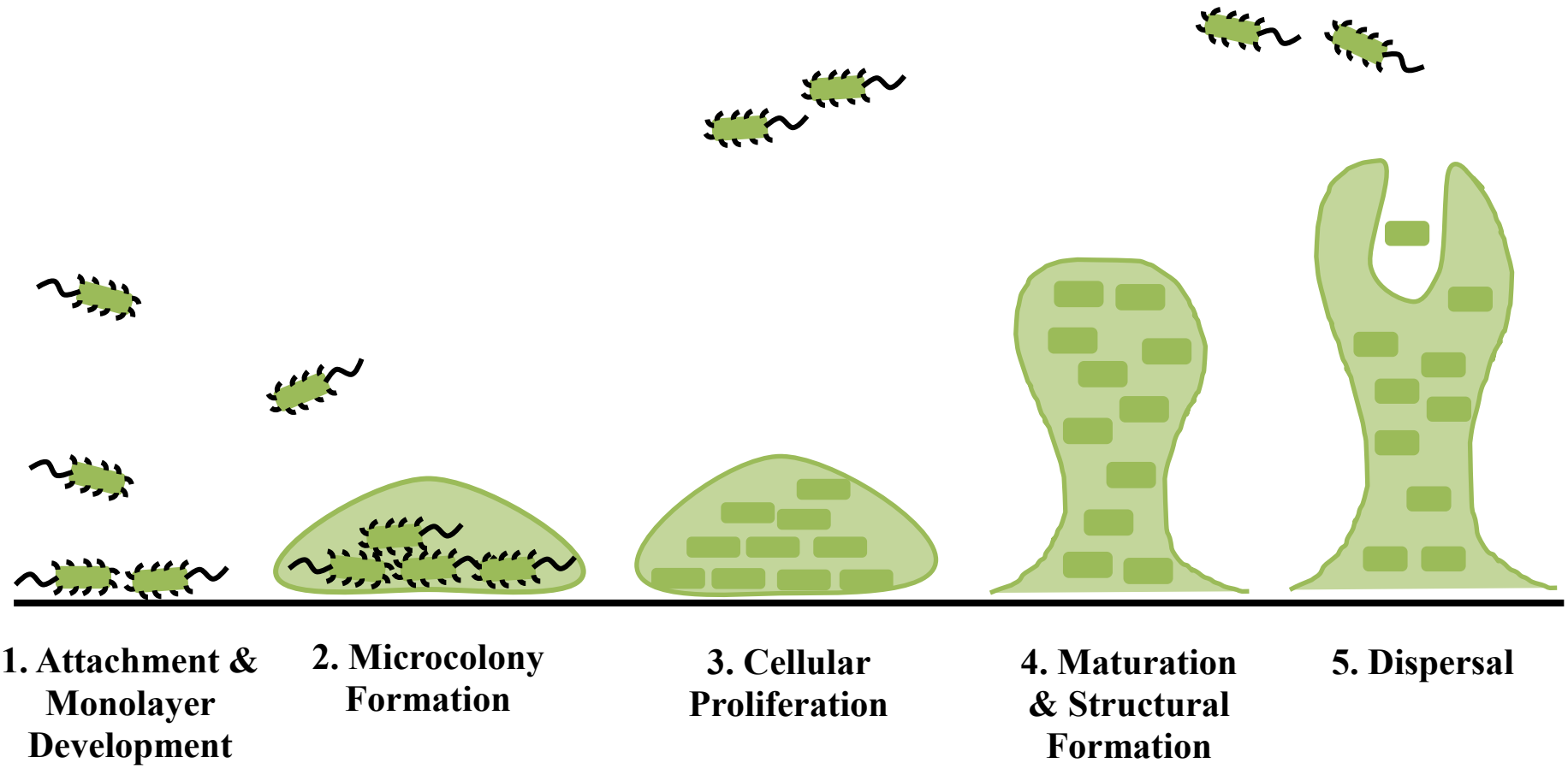


Figure 1.4 The five developmental stages involved in biofilm formation.

1.3.4.1 Biofilm Models

Depending on the model being utilised, bacterial adhesion, biofilm structure, or both factors can be determined *in vitro*. Nevertheless, it should be noted that the term 'biofilm' is often used to describe both modes of growth within a community formation. The two most commonly used methods for the investigation of bacterial 'biofilm' growth, are the microtiter plate based assay and the flow cell model (Azeredo *et al.* 2017).

The static microtitre plate based assays is a highly reproducible and repeatable model which allows for rapid, high throughput screening of bacterial adhesion capacity under different atmospheric conditions using standardised experimental parameters (O'Toole 2011). Adhesion of the bacterial cells to the walls of the wells in a 96 well microtitre plate can be quantified using a plate reader to determine optical density following a simple stain and decolourisation technique. (O'Toole 2011; Stepanovic *et al.* 2007). This methodology does not allow the assessment of bacterial community and structural formation.

Alternatively, the flow cell model is considered a "gold standard" assay which facilitates the visualisation of substratum bacterial adhesion, biofilm formation and maturation under hydrodynamic conditions. This model requires specialised equipment and expertise, and owing to several technical aspects, this methodology faces some issues with variability and reproducibility. Nevertheless, standardisation of various technical aspects can reduce this variability (Heydorn *et al.* 2002; Heydorn *et al.* 2000). This method allows for factors such as nutrient availability, the introduction of supplements and antimicrobials, medium flow rate, time and temperature to be altered and assessed. Depending on the organism being tested and the operational parameters applied, the flow cell model can run over many days. In contrast to the microtitre plate based assay, this model allows the examination of complex biofilm structures in the absence of planktonic cells which are removed by the media flow. Collection of effluent for assessment of dispersed cell morphology including the development of small colony variants is also easily achieved with this model. Visualisation of the three dimensional (3D) biofilm structure is undertaken using confocal laser scanning microscopy (CLSM), thus allowing non-invasive analysis in real time. Introduction of fluorescent stains prior to microscopy, or the use of fluorescently tagged bacteria, can reveal the composition of the biofilm, the ratio of live to dead cells, and allow the determination of seeding dispersal (Crusz *et al.* 2012; Kirov *et al.* 2007). Results from the CLSM can be analysed using specialised Comstat software which allows assessment of various parameters including biomass, thickness and surface area (Comstat2 2015; Heydorn *et al.* 2000; Voorregaard 2008). While much of the literate surrounding bacterial biofilm formation utilises high throughput screening assays, there is a lack of evidence-based science

that confirms the correlation of these methodologies to those which assess biofilm structural development. Inherent design differences make these comparisons difficult. Commonly utilised biofilm devices such as the microtitre plate assay and the Calgary device have been designed to assess the biofilm forming capacity of cells. Both techniques are screening methods which allow for high throughput analysis and incubation under varied growth conditions. Results generated are easily interpreted; however, they may be limited due to loosely adhered cells being removed during washing and cellular death following exhaustion of nutrients. In contrast, the flow cell apparatus allows for the visualisation of biofilm development and structure. Experimental conditions can be altered to assess growth under antibiotic pressure. While this device can be optimised more accurately mimic in vivo conditions, it requires specialised equipment and technical expertise to undertake and interpret (Azeredo *et al.* 2017). Each of the different biofilm devices have been designed to assess different aspects of development and as a result there is little consensus between these techniques and their interpretation. Furthermore, there is limited data presented which associate in vitro biofilm capabilities with clinical outcomes for people with CF. Previously, much work has been undertaken that describes the phenotypic and biofilm characteristics of isolates obtained from patients with CF. Furthermore, genetic analysis has demonstrated a number of mutations which directly impact the cells ability to be motile, adhere and disperse. However, due to limitations in experimental design the translation of in vitro results to in vivo impact remains incomplete.

1.4 Research Aims and Objectives

1.4.1 Background and Scope

P. aeruginosa is the most commonly isolated bacterial pathogen from respiratory secretions obtained from patients with CF. Intermittent infection with *P. aeruginosa* is often seen in childhood, while by adulthood many patients have developed chronic infection (Bell *et al.* 2011; Cystic Fibrosis Australia 2015). Early infecting strains, thought to be primarily acquired from environmental sources, are usually motile, susceptible to antipseudomonal antibiotics and exist in a free-living state (Luzar *et al.* 1985; Smith *et al.* 2006). These early infecting strains, which typically cause intermittent infection, can be cleared by activation of host immune pathways or by antibiotic treatment (Gibson *et al.* 2003). Once a strain becomes established within the lower airway, it begins to adapt to the CF lung and eradication is no longer possible.

Adaptation of *P. aeruginosa* involves a cascade of physiological, metabolic and morphological changes which affect their mode of growth, expression of virulence factors and interactions with host immune cells, resulting in chronic infection (Smith *et al.* 2006). Genomic studies have described the presence of a number of genes and regulatory pathways which are thought to play a significant role in loss of virulence factors and adaptation of *P. aeruginosa* to the environment (Marvig *et al.* 2015; Winstanley *et al.* 2016). Important adaptive changes include loss of motility, tolerance to decreased oxygen levels, and the establishment of complex biofilm communities (Hogardt and Heesemann 2013; Hoiby *et al.* 2011).

The scientific literature details many studies describing the motility and biofilm forming characteristics of *P. aeruginosa* isolated from patients with CF (Carter *et al.* 2010; Clark *et al.* 2015; Cullen *et al.* 2015; Fothergill *et al.* 2012; Grosso-Becerra *et al.* 2014; Head and Yu 2004; Jiricny *et al.* 2014; Kukavica-Ibrulj *et al.* 2008; Manos *et al.* 2013; O'May *et al.* 2006). Overall, these studies demonstrate that during chronic infection, *P. aeruginosa* adopts a biofilm mode of growth, but there appears to be some variability in each strain's biofilm forming capacity. Likewise, there seems to be some inter-strain variation with respect to motility (Jeukens *et al.* 2014; Winstanley *et al.* 2009). However, it must be noted that in most of these studies the overall number of isolates (in particular, those of environmental origin) tested and the diversity of the strains used was limited. Typically, most studies have relied on using the PAO1 strain or knockout mutants as comparators (Carter *et al.* 2010; Jeukens *et al.* 2014; Jiricny *et al.* 2014; Kukavica-Ibrulj *et al.* 2008; Manos *et al.* 2008; Manos *et al.* 2013; O'May *et al.* 2006; Yang *et al.* 2011). While a standard needs to be included to account for inter-run variation and to set a baseline for isolate comparison, the expression and capabilities of this strain needs to be known in the context of the broader population so that degrees of change from this

baseline can be interpreted correctly. When a comparison to a known negative (such as a mutant) is made, care also needs to be taken when determining the level of positivity assigned to a strain, as next to a true negative, most isolates will appear positive.

In some instances the fundamental principles underpinning bacterial adhesion and initial microcolony development (i.e. functioning cell surface organelles, flagella and pili) also remain uncertain (Proft and Baker 2009; Stoodley *et al.* 2002). Again, these differences may simply reflect inter-strain variability; however, most studies have utilised poorly characterised strains of an unknown genetic background. Other sources of variability could be associated with methodological inconsistencies, including the use of experimental conditions (e.g. reduced temperature and oxygen availability), which do not reflect those encountered in the CF airway.

Epidemiological investigations have demonstrated that patients with CF can share indistinguishable strains of *P. aeruginosa*, and it is speculated that acquisition occurs by patient-to-patient transmission (Knibbs *et al.* 2014; Wainwright *et al.* 2009). In Queensland, the predominant shared strain is AUST-02 (Kidd *et al.* 2013). Ecological studies to date have failed to find this strain in any environmental setting apart from respiratory secretions collected from patients with CF (Kidd *et al.* 2012). In 2007/2008, AUST-02 was the predominant strain isolated from 40% of patients with a confirmed *P. aeruginosa* infection living in Queensland, Australia. This strain is of particular importance given its potential for patient-to-patient spread, lack of environmental reservoir and association with increased hospitalisation and treatment requirements (Kidd *et al.* 2013; Knibbs *et al.* 2014). Currently little is known about the phenotypic and genotypic characteristics of the AUST-02 strain as many of the previous studies have focused on the shared CF strains, LES and AUST-01, prevalent in Europe and Australia, respectively. This work has described these strains as strong biofilm producers when compared to PAO1 (Carter *et al.* 2010; Fothergill *et al.* 2012; Manos *et al.* 2008).

Therefore, to improve understanding of the adaptive biology of *P. aeruginosa* and, more specifically, the AUST-02 strain, a large component of this PhD thesis is devoted to defining the motility and biofilm-forming capacity of numerous isolates selected from a well characterised biobank (Kidd *et al.* 2012). The isolates in the biobank have been genotyped using MLST, providing the opportunity to study the phenotypic and genomic differences between isolates from similar and distant genetic backgrounds. In addition, access to multiple isolates of the same ST that have been cultured from different ecological settings (e.g. ST-155, ST-179, ST-242, ST-266, ST-381), may help to explain why some *P. aeruginosa* strains are more common and appear to have an enhanced capacity to adapt and survive in new environments.

While the majority of *P. aeruginosa* strains are capable of surviving in various ecological niches, properties such as patient-to-patient transmissibility and adaptation to specific hosts (i.e. patients with CF), are features of the AUST-02 strain. Therefore, comparing AUST-02 with other closely related CF (non-shared), non-CF, animal and environmental strains may provide the key to identifying specific adaptations that allow transmission and persistence to occur.

To better understand these processes and to add to the existing knowledge of bacteria adaptation, during this PhD project I will focus on adhesion, motility and biofilm formation of *P. aeruginosa* isolates obtained from a range of clinical and environmental settings, under varied atmospheric conditions. Furthermore, I will be ascertaining if AUST-02 possesses any of the aforementioned adaptive mechanisms and determining how they may be relevant in the establishment and persistence of airway infection in people with CF. Finally, through the use of whole genome sequencing and bacterial genome-wide association study I will aim to further comment on the diversity of the strains studied and determine if any mutations identified can explain the phenotypic characteristics described.

National CF data registries collate and present clinical, microbiological and demographic data on patients (Cystic Fibrosis Australia 2016; Cystic Fibrosis Foundation 2015; Cystic Fibrosis Trust 2016). While the broad overview provided by the registries captures changing trends over time, due to their large scale, the fine nuances of the impact of antibiotic exposure, acquisition of new bacteria or changing bacterial genotypes within patient cohorts are not able to be presented. Studies which focus on the specific epidemiological trends of a single centre, or defined groups of patients, are better able to comment on emerging pathogens, diversity within individual species, changing acquisition rates and the health outcomes associated with these events (Kidd *et al.* 2015; Millar *et al.* 2009; O'Carroll *et al.* 2004; Spicuzza *et al.* 2009; Valenza *et al.* 2008). By doing so, surveillance studies using defined patient cohorts, conducted within clinics following recognised standards of care, may generate results which can be extrapolated into the wider CF community. In this PhD project I will i) describe the changing prevalence of relevant bacterial and fungal pathogens identified from sputum samples collected from a local adult CF centre, ii) genotype *P. aeruginosa* isolates collected during two cross-sectional studies iii) identify new acquisitions of shared strains within this cohort, and iv) determine if clinical decline is associated with acquisition.

This thesis aims to provide a detailed microbiological epidemiological surveillance of both the bacterial species and strain diversity of patients attending a single CF specialist centre. In addition,

health outcomes, effectiveness of antibiotic treatment and the robustness of a research-based definition used to categorise chronic infections will be assessed and validated within a dynamic clinical setting. Furthermore, by determining the fundamental biological differences which may exist between shared strains and those readily isolated from numerous settings, improvements in preventing acquisition, eradicating infections and ongoing treatments may be developed.

1.4.2 Broad Research Aims

This thesis aims to determine the longitudinal prevalence and diversity of *P. aeruginosa* within an adult CF clinic and through comparison with non-CF and environmental isolates, identify important biological characteristics in the context of CF airway infection and cross-infection.

1.4.3 Specific Research Aims

The specific aims of the research describe in this thesis were to:

1. To determine the change in prevalence over time for *P. aeruginosa* and a range of other significant CF pathogens in a local CF population.
2. To determine the impact of Pseudomonas eradication therapy on patients at time of transition into the adult CF centre.
3. Validate the robustness of the 'Leed's Criteria' for defining chronic *P. aeruginosa* in a dynamic adult CF centre.
4. To determine the incidence and prevalence of shared strains in a local CF population using isolates collected at two defined time points.
5. To assess the clinical impact following acquisition of a shared *P. aeruginosa* strain.
6. Through the use of phenotypic assays, identify the adaptation mechanisms of a large number of well characterised *P. aeruginosa* isolates with particular focus on the comparison of:
    a. Isolates from patients with CF and the environment,
    b. Isolates that share close evolutionary relationships and,
    c. Genetically indistinguishable isolates.
7. Determine if the high throughput rapid plate-based adhesion assay can be used interchangeably with (or even replace) the flow cell as an indicator of biofilm forming capacity.
8. Undertake bioinformatic analysis on a subset of isolates to identify genetic variants responsible for phenotypic characteristics.

Chapter 2: Prevalence of *Pseudomonas aeruginosa* in an adult CF centre

2.1 Abstract

Background: Increased patient longevity and aggressive antibiotic treatment are thought to impact on the microbial composition of the airways of adults with cystic fibrosis (CF). In this study we sought to determine if a temporal change in the airway microbiology of adults with CF has occurred over time.

Methods: Longitudinal analysis of sputum microbiology results was undertaken on patients attending a large adult CF centre. Clinical status and health outcomes of transitioning patients were also assessed.

Results: A decrease in the prevalence of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia cepacia* complex and *Aspergillus spp.* ($P = 0.001$, $P < 0.001$, $P = 0.002$ and $P < 0.001$, respectively) occurred. Improvements in lung function among transitioning patients infected with *P. aeruginosa* were observed.

Conclusion: Overtime a decline in the prevalence of many CF airway pathogens has occurred. Significantly an incremental improvement in lung function was reported for transitioning patients with current *P. aeruginosa* infections.

2.2 Introduction

Cystic fibrosis (CF) is a multisystem disease, in which a great burden of the morbidity and mortality results from chronic suppurative lung disease. Pathophysiologically the disease is characterised by dehydration of the airway surface liquid, elevated mucus production and increased susceptibility to infections (O'Sullivan and Freedman 2009; Ratjen *et al.* 2010). A number of microorganisms are associated with CF airway infections, the prevalence of which varies according to patient age (Chmiel *et al.* 2014; Lipuma 2010; O'Sullivan and Freedman 2009). *Haemophilus influenzae* and methicillin-sensitive *Staphylococcus aureus* (MSSA) are frequently the earliest pathogens isolated in children and this is often (Salsgiver *et al.* 2015; Spicuzza *et al.* 2009; Valenza *et al.* 2008) followed by *Pseudomonas aeruginosa* (Chmiel *et al.* 2014; Lipuma 2010; O'Sullivan and Freedman 2009). Early infection in children can progress to a chronic infection where eradication cannot be achieved. So, early treatment aims to prevent or delay the time to chronic infection. Aggressive antibiotic therapy is recommended at the time of *P. aeruginosa* acquisition and is usually successful in achieving initial eradication (Doring *et al.* 2012; Langton Hewer and Smyth 2014; Mayer-Hamblett *et al.* 2015). Programs aimed at eradicating early *P. aeruginosa* infection have been widely adopted over the past two decades with rates of *P. aeruginosa* infection progressively declining in young people especially in children and adolescents with CF (Cystic Fibrosis Trust 2015; Cystic Fibrosis Trust 2006; Razvi *et al.* 2009; Salsgiver *et al.* 2015). It is less clear what impact these early changes are having on adult populations on rates of *P. aeruginosa* and other pathogens such as nontuberculous mycobacteria (NTM) infection, methicillin-resistant *S. aureus* (MRSA) and emergent bacteria (*Stenotrophomonas maltophilia* and *Achromobacter spp*.) (Cystic Fibrosis Australia 2015; Cystic Fibrosis Foundation 2015; Cystic Fibrosis Trust 2015; Millar *et al.* 2009; Salsgiver *et al.* 2015; Valenza *et al.* 2008).

P. aeruginosa remains the most prevalent microorganism isolated from adults with CF (Cystic Fibrosis Australia 2015; Cystic Fibrosis Foundation 2015; Cystic Fibrosis Trust 2015; Lipuma 2010; Millar *et al.* 2009; Salsgiver *et al.* 2015; Valenza *et al.* 2008). Once established *P. aeruginosa* infections are difficult to eradicate and classically have been associated with accelerated lung function decline and poorer survival (Emerson *et al.* 2002). Several studies have defined the term, chronic infection, to provide a basis for diagnosing persistent infection (Lee *et al.* 2003; Pressler *et al.* 2011). Despite the ongoing debate about a strict definition to describe chronic *P. aeruginosa* infection, this term is now widely used including in some CF Data Registries to stratify infection status. Clinically this definition may guide the physician in determining a therapeutic approach for the patient. However, the utility of these definitions

in clinical practice is limited by the inability of some patients to provide sufficient sputum samples within a given time period, inadequate sample quality and limited follow-up review. Furthermore, such criteria are yet to be validated in adults with CF.

The aims of this study were to determine the prevalence of infection in an adult population of patients with CF and to assess the utility of the Leed's criteria in adults within a clinical setting to define chronicity of *P. aeruginosa* (Lee *et al.* 2003). Furthermore, we were particularly interested in the clinical and microbiological characteristics of patients, but due difficulties associated with patient movement into and from this clinic (death, transplant and transfer to another care centre), the clinical impact of changing pathogen prevalence was reported only for those patients transitioning to adult care to determine changes occurring during the modern era of eradication therapy.

2.3 Methods

2.3.1 Study population

All patients with CF attending The Prince Charles Hospital (TPCH) Adult CF Centre located in Queensland, Australia, between 1st January 2001 and 31st December 2014 were included in the study. Clinical measurements were excluded following transplantation. The centre provides care for Queensland, the Northern Territory and northern New South Wales adults with CF, with national standards of care recommending a minimum of four multi-disciplinary outpatient reviews per year (Bell and Robinson 2008).

In 2013, there was a median of six (interquartile range [IQR]: 2 - 11) outpatient attendances at TPCH Adult CF Centre. Sputum samples were collected at each review and at the beginning and completion of all intravenous antibiotic treatment courses (inpatient and or hospital-in-the-home). Expectorated sputum was primarily collected with induced sputum sampled in selected non-productive patients with unexplained clinical decline (total 3260 samples from 469 patients). Induced sputum samples were unable to be differentiated from expectorated sputum as both were coded in the same way. Bronchoscopy samples were rarely collected and usually when unexplained clinical decline had occurred. During the study period, 51 bronchoscopic samples (bronchoalveolar lavage and bronchial washings) from 34 patients were collected representing 1.5% of the total samples submitted for culture. The sampling frequency from bronchoscopy samples did not change over time. Upper airway samples for bacteriological analysis were not routinely performed. This study was approved by TPCH, Human Research Ethics Committee (HREC/13/QPCH/51).

2.3.2 Sample collection and microbiological analysis

Only results from sputum sample processing were included in this study. Sputum culture and microbiological identification was undertaken by a routine diagnostic laboratory using a combination of best practice standard phenotypic and molecular techniques (Gilligan *et al.* 2006). Apart from the introduction of bacterial identification using the bioMerieux VITEK® MS (Mass spectrometry microbial identification system) in 2011, all other isolation and identification techniques remained consistent.

All fungal species reported were grown on routine bacterial culture media and specific NTM testing using standard solid and liquid culture media for mycobacteria were undertaken in the Mycobacterial

Reference Laboratory when requested by the treating physician (i.e. annually, when clinically suspected or when NTM had previously been cultured).

2.3.3 Microbiological and clinical data collection

Sputum microbiology results were obtained electronically from the Pathology Queensland Clinical and Scientific Information System (AUSLAB). In the first instance clinical data were obtained from the TPCH CF patient database and hospital medical records, and if unavailable, information from either the Australian CF Data Registry or the referring CF centre at the time of transition were used. Notably, centres enter clinical and microbiological data into the Australian CF Data Registry on an encounter-basis allowing the capture of all sputum samples collected in each year. Of the 217 transitioning patients, sputum microbiological results were obtained from letters provided at the time of health-care facility transition (n = 6), the Australian CF Data Registry (n = 13) and the TPCH CF patient database (n = 198).

Due to yearly fluctuations in patient numbers, assessing the clinical characteristics of all patients at one given time was not possible with this data; therefore, the clinical characteristics were presented for transitioning patients at time of entry to TPCH. Furthermore, the impact of reduced pathogen rates in younger, transitioning patients on prevalence rates of specific pathogens in the whole clinic will be 'diluted' by the proportion of existing patients with chronic infection with common CF pathogens (e.g. *P. aeruginosa*) only.

Age-adjusted pulmonary function using recognised prediction equations (Hankinson *et al.* 1999; Wang *et al.* 1993), and standard deviation *z*-scores for body mass index (BMI) (calculated using United States National Centre for Health Statistics and Centres for Disease Control and Prevention normalised growth reference values) were determined for all patients aged two to 20 years. Patients older than 20 years at the time of transition were removed from analysis (n = 14). Cystic fibrosis transmembrane conductance regulator (CFTR) function was defined as minimal function (Class I, II or III mutations), residual function (≥ 1 allele with a Class IV or V mutation) and unknown function (Clinical and Functional Translation of CFTR (CFTR2) ; Cystic Fibrosis Foundation 2014; Green *et al.* 2010).

Patients attending TPCH on the first occasion were defined as new patients and were stratified into one of three groups: i) transitioning patients that had their care transferred from a paediatric CF Centre (n =

217), ii) transferring patients from another adult CF Centre (n = 107), and iii) adults diagnosed with CF for the first time (n = 12).

Patient infection status was also stratified according to those: i) with chronic *P. aeruginosa* infection, ii) in whom *P. aeruginosa* infection status was intermittent, iii) with no *P. aeruginosa* infection, and iv) where no sputum sample was collected. The Leed's criteria (Lee *et al.* 2003) were used for the initial assessment of chronic *P. aeruginosa* infection. If a patient could not meet the definition due to an insufficient number of cultures, then a modified definition was used.

Chronic *P. aeruginosa* infection in patients providing four or more sputum samples in a calendar year was determined when > 50% of sputum samples were culture positive exactly as for the Leed's criteria. The key modification was in instances where fewer than four samples were collected or less than 50% of samples were culture positive; data from the previous year was reviewed and if the patients also fulfilled the above criteria they were also classified as having chronic infection.

In cases where insufficient information allowed a patient to be defined as chronic, the patient was classified as having an intermittent *P. aeruginosa* infection. If a patient was not seen at the CF centre in a specific calendar year their data was not included for that year.

To account for a relatively small number of transitioning patients per annum, the study period was divided into tertiles (i.e. 2001 - 2004, 2005 - 2009, 2010 - 2014). Prevalence of each microorganism was defined for each patient where at least one positive culture result was available within a calendar year.

2.3.4 Statistical analyses

SPSS version 22 was used for statistical analysis. Associations for categorical variables were examined using the Pearson Chi-squared test and Fisher's Exact test when more than 20% of the expected values were less than five. If the *P* value was significant ($P \leq 0.05$) pairwise comparisons between each of the tertile periods were further examined. Continuous variables were examined using the Mann-Whitney U test. To assess if lung function was dependent on *P. aeruginosa* status and year of entry into the adult CF centre (n = 469), linear regression models for forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) percent predicted (%pred) containing terms *P. aeruginosa* status, year of entry and their interaction were examined. As the interaction term was significant a one-way analysis of variance

(ANOVA) of FEV_1 and FVC by year of entry, stratified by *P. aeruginosa* status was used to further explore the interaction. Where the global ANOVA *P*-value was significant ($P \leq 0.05$) a least significant difference *post hoc* test was completed to detect which cohort years were different for *P. aeruginosa* status.

2.4 Results

2.4.1 Study Population

Over the 14-years a total of 469 individuals (n = 3260 patient years) attended TPCH Adult CF Centre including 336 new patients, which consisted of 217 transitioning patients, 107 transferring patients and 12 patients who were diagnosed in adulthood (Figure 2.1). Seventy-eight patients underwent lung transplantation (male = 34) and 49 died (male = 22) during the study period, with a median of four years attendance for clinical review at TPCH prior to transplant or death. Patients were typically young adults and they remained at the centre for median six years and provided a median of three sputum samples annually (Table 2.1).

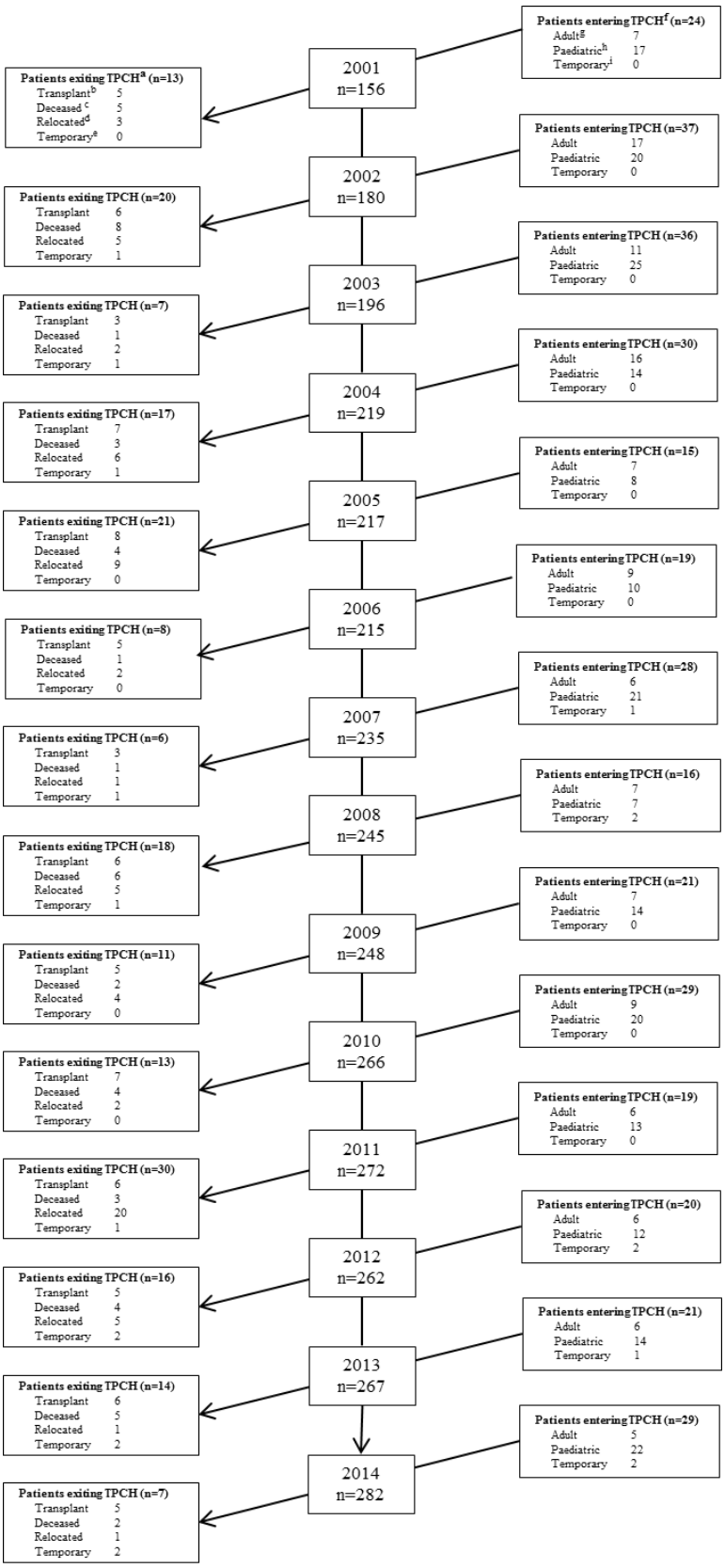


Figure 2.1 Patient movement occurring at The Prince Charles Hospital Adult Cystic Fibrosis Centre between 2001 and 2014.

[a] Patients exiting TPCH; Includes all patients leaving The Prince Charles Hospital Adult Cystic Fibrosis Centre during the calendar year

[b] Transplant; Patients who have undergone lung transplantation during the calendar year

[c] Deceased; Patients who have died during the calendar year

[d] Relocated; Patients who have transferred to another CF centre during the calendar year

[e] Temporary; Patients who have temporary left The Prince Charles Hospital Adult Cystic Fibrosis Centre during the calendar year

[f] Patients entering TPCH; Includes all new patients arriving to The Prince Charles Hospital Adult Cystic Fibrosis Centre during the calendar year

[g] Adult; Adult transfer patients entering The Prince Charles Hospital Adult Cystic Fibrosis Centre and patients newly diagnosed as adults during the calendar year

[h] Paediatric; Transitioning patients entering The Prince Charles Hospital Adult Cystic Fibrosis Centre during the calendar year

[i] Temporary; Patients who have temporary returned to The Prince Charles Hospital Adult Cystic Fibrosis Centre during the calendar year

Table 2.1 Characteristics of the 469 patients attending The Prince Charles Hospital Adult Cystic Fibrosis Centre between 2001 and 2014.

Patient Characteristics	
Age at initial review; median years (IQR) [a]	20.0 (18.0-27.0)
Gender; no. of males (%)	242 (51.6)
Deaths; no. (%)	49 (10.4)
Age at death; median years (IQR)	28.3 (20.8-37.2)
Lung transplantation; no. (%)	78 (16.6)
Age at transplantation; median years (IQR)	29.8 (25.0-37.5)
Years attending TPCH; median (IQR)	6.0 (2.5-11.0)
Annual sputum collection; median (IQR)	3.0 (1.0-6.0)

[a] The Prince Charles Hospital Adult Cystic Fibrosis Centre (transitioning from paediatric care or transferring from another adult care centre)

2.4.2 Infection Prevalence

One or more CF pathogens were isolated in 98% (2678/2735 patient years) of all patients able to produce sputum samples. There was a significant decrease in prevalence over time for total *P. aeruginosa* infection ($P = 0.001$), MSSA ($P < 0.001$), MRSA ($P < 0.001$), *B. cepacia* complex ($P = 0.002$) and *Aspergillus spp.* ($P < 0.001$) isolation (Table 2.2). Thirty-four patients underwent 51 bronchoscopy procedures with the annual number similar over the study period, rates of *P. aeruginosa* infection were lower in this cohort (56.9% of samples, compared with 74.7% of all sputum samples, $P = 0.006$) and rates of NTM infection were not significantly different (7.8% of samples, compared with 4.0% of all sputum samples, $P = 0.150$).

Table 2.2 Tertile groupings by years of infection prevalence for the 469 patients attending The Prince Charles Hospital Adult Cystic Fibrosis Centre between 2001 and 2014, n (%).

Year	Number of patient years	*Pseudomonas aeruginosa*		*Staphylococcus aureus*		Achromobacter spp.	*S. maltophilia*	*B. cepacia* complex	Aspergillus spp.	Scedosporium spp.	NTM [%]	No pathogen detected	No sample collected
		Positive	Chronic[$]	MSSA	MRSA								
2001-2004	751	574 (76.4)[a]	488 (65.0)	302 (40.2)[a]	62 (8.3)	53 (7.1)	53 (7.1)[b]	63 (8.4)	227 (30.2)[a]	13 (1.7)	22 (2.9)	131 (17.4)[ab]	113 (15.0)[a]
2005-2009	1160	900 (77.6)[a]	796 (68.6)	457 (39.4)[a]	53 (4.6)	67 (5.8)	112 (9.7)	67 (5.8)[c]	313 (27.0)[a]	29 (2.5)	48 (4.1)	171 (14.7)[a]	157 (13.5)[a]
2010-2014	1349	962 (71.3)	907 (67.2)	394 (29.2)	38 (2.8)	68 (5.0)	88 (6.5)[b]	63 (4.7)[c]	254 (18.8)	32 (2.4)	64 (4.7)	280 (20.8)[b]	255 (18.9)
Total	3260	2436 (74.7)	2191 (67.2)	1153 (35.4)	153 (4.7)	188 (5.8)	253 (7.8)	193 (5.9)	794 (24.4)	74 (2.3)	134 (4.1)	582 (17.9)	525 (16.1)
P value*		0.001	0.250	< 0.001	< 0.001	0.160	0.010	0.002	< 0.001	0.520	0.130	< 0.001	0.001

[a, b, c] numbers within columns with a letter in common are not significantly different.

* Significant decrease in *P. aeruginosa*, MSSA, MRSA, *B. cepacia* complex and Aspergillus infections and a significant increase in *Stenotrophomonas maltophilia* infection.

MSSA: methicillin sensitive *Staphylococcus aureus*; MRSA: methicillin resistant *Staphylococcus aureus*; *S. maltophilia*: *Stenotrophomonas maltophilia*; NTM: nontuberculous mycobacteria.

[%] Nontuberculous mycobacteria species: *M. abscessus* (n = 66), *M. avium* (n = 8), *M. bolletii* (n = 1), *M. chelonae* (n = 2), *M. fortuitum* (n = 3), *M. intracellulare* (n = 55), *M. lentiflavium* (n = 1), *M. massilense* (n = 1), *M. scrofulaceum* (n = 2), *M. shimoide* (n = 1), *M. simiae* (n = 1), *M. species* (n = 1); seven patients had more than one NTM infection.

[$] A modification of the Leed's criteria was used to define chronic *P. aeruginosa* infection: i) in patients providing four or more sputum samples in a calendar year was determined when > 50% of sputum samples were culture positive and ii) in instances where fewer than four samples were collected or less than 50% of samples were culture positive; data from the previous year was reviewed and if the patients also fulfilled the above criteria they were also classified as having chronic infection.

2.4.3 Leed's Criteria in an Adult CF Centre

Over the period 2001 to 2014, a total of 3262 patient-years were studied and a median of three (IQR: 1 - 6) sputum samples per patient per year were collected. When patients who did not provide any samples in a year were excluded, a median of four (IQR: 2 - 7) samples were collected. Of these patients, those who provided $\geq$ 4 samples represented 1441 of 2735 patients-years (53%).

To illustrate the impact of applying the Leed's versus the Modified Leed's criteria for defining chronic *P. aeruginosa* infection, data from 2013 and 2014 were compared using both definitions. Using the Leed's criteria to define patients with chronic *P. aeruginosa* infection ($\geq$ 50% of $\geq$ 4 samples analysed) in 2013, 190 patients were identified, yet if we then examined the same cohort using the prior year (2012), an additional 25 patients were identified (Table 2.3A). Similarly, in examining the 2014 data and applying the Leed's criteria, we identified 193 patients and then when we applied the extended year criteria, 28 additional patients were identified (Table 2.3B). Using the modified Leed's criteria, 67% of CF adults had chronic *P. aeruginosa* infection and this prevalence remained stable over time ($P = 0.250$).

Table 2.3 Comparisons of Leeds Criteria with the Modified Criteria to define *Pseudomonas aeruginosa* infection status [a].

Panel A

YEAR 2013 - TOTAL PATIENTS = 267

	Chronic *P. aeruginosa* infection	Indeterminant *P. aeruginosa* infection	No *P. aeruginosa* isolated		No sample collected	
Leed's Criteria	190	6	28		43	
Modified Criteria	215	6	No *Pa* isolated [b]	Chronic infection	No sample [c]	Chronic Infection
			19	9	27	16

Panel B

YEAR 2014 - TOTAL PATIENTS = 282

	Chronic *P. aeruginosa* infection	Indeterminant *P. aeruginosa* infection	No *P. aeruginosa* isolated		No sample collected	
Leed's Criteria	193	6	36		47	
Modified Criteria	221	6	No *Pa* isolated [b]	Chronic infection	No sample [c]	Chronic Infection
			25	11	30	17

[a] A modification of the Leed's criteria was used to define chronic *P. aeruginosa infection*: i) in patients providing four or more sputum samples in a calendar year was determined when > 50% of sputum samples were culture positive and ii) in instances where fewer than four samples were collected or less than 50% of samples were culture positive; data from the previous year was reviewed and if the patients also fulfilled the above criteria they were also classified as having chronic infection.

[b] No *Pa* isolated: No *P. aeruginosa* isolated

[c] No Sample: No sample collected

2.4.4 Transitioning Patients

A total of 217 transitioning patients were identified moving into adult care over the 14 years, with between seven and 25 new patients (median: 14) annually. There were no differences in the mean patient age at entry (P = 0.350), distribution of CFTR genotypes (P = 0.740) or proportion of patients with pancreatic insufficiency (P = 0.270). We observed significant improvements in lung function (FEV_1 %pred P = 0.008; FVC %pred; P < 0.001) and nutritional status (P = 0.048) and an increase in patients diagnosed with CF-related diabetes (P = 0.031) over time (Table 2.4). Improvements in lung function appeared to be dependent upon *P. aeruginosa* infection status (Table 2.5). There was a significant and incremental improvement in lung function across cohorts for patients with *P. aeruginosa* infection.

Overall, we observed that there was a 13% increase in the number of patients unable to provide a sputum sample, and an 8% reduction in organisms isolated when comparing the 2010 - 2014 cohort with the 2001 - 2004 cohort (Table 2.6). *P. aeruginosa*, MSSA and *Aspergillus spp.* represented the most common pathogens detected in transitioning patients. At the time of transition, *P. aeruginosa* was cultured from 138/217 (64%) patients, with 80% of these patients suffering chronic infection (110/138). Over the 14 years, there was a significant decrease in the prevalence of total *P. aeruginosa* (P = 0.010). The frequency of chronic *P. aeruginosa* infection also decreased, with patients transitioning between 2010 - 2014 17% less likely to have chronic *P. aeruginosa* (43%) than those who transitioned in the 2001 - 2004 cohort (61%), though this was not statistically significant (P = 0.087). We also observed an increase in the prevalence of *S. maltophilia* (P = 0.019), while the prevalence of other CF pathogens did not change significantly over cohorts.

Table 2.4 Clinical characteristics of the 217 transitioning patients at the time of first review at The Prince Charles Hospital Adult Cystic Fibrosis Centre.

	2001 to 2004 n = 76	2005 to 2009 n = 60	2010 to 2014 n = 81	*P* value
Sex, males, n (%)	46 (60.5)	29 (48.3)	36 (44.4)	0.120
Age at transition, years, mean (SD)	17.6 (1.7)	17.7 (1.4)	17.9 (0.9)	0.350
CFTR genotypes; n (%) [a]				0.740
Two Class I-III mutations	57 (78.1)	42 (71.2)	59 (73.8)	
One or two Class IV or V mutations	3 (4.1)	6 (10.2)	6 (7.5)	
Non classified	13 (17.8)	11 (18.6)	15 (18.8)	
CF related diabetes; n (%)	4 (5.3)	6 (10.0)	15 (18.5)	0.031
Pancreatic insufficiency; n (%)	72 (94.7)	53 (88.3)	71 (87.7)	0.270
BMI *z*-score; median (IQR) [b,c]	19.4 (17.8-21.4)	20.1 (18.6-22.2)	20.2 (19.0-22.9)	0.048
FEV_1 % predicted; mean (SD) [d,e]	64.5 (24.6)	66.9 (20.7)	75.1 (20.5)	0.008
FVC % predicted; mean(SD) [d,e]	74.7 (22.4)	77.2 (18.6)	87.0 (16.3)	< 0.001

[a] CFTR genotypes defined Green *et al*. No CFTR genotype available on 5 patients, therefore not included (n = 212)

[b] BMI percentiles for boys and girls (2 - 20 years)

[c] Missing data on one patient; 14 patients > 20 years, therefore *z*-score not calculated (n = 202)

[d] Calculated using Hankinson *et al* 1999 and Wang *et al* 1993.

[e] Missing data on one patient (n = 216)

Table 2.5 Change in lung function parameters over time when compared to year of entry and *Pseudomonas aeruginosa* status for 217 transitioning patients at time of first review at The Prince Charles Hospital Adult Cystic Fibrosis Centre.

		Year of Entry [*]			
P. aeruginosa status	Outcome	2001 to 2004 [#]	2005 to 2009 [^]	2010 to 2014 [$]	*P* value (all years)
Positive	FEV_1 % Predicted, mean (95% CI)	59.1 (53.6 - 64.7) [a]	66.0 (59.1 - 72.9) [ab]	73.6 (67.2 - 80.0) [b]	0.006
Chronic		55.6 (49.5 - 61.8) [a]	64.0 (56.3 - 71.7) [ab]	71.6 (64.6 - 78.6) [b]	0.008
Positive	FVC % Predicted, mean(95% CI)	70.1 (65.3 - 74.9) [a]	75.7 (69.7 - 81.8) [a]	86.3 (80.7 - 91.9)	< 0.001
Chronic		66.9 (61.6 - 72.3) [a]	74.5 (67.8 - 81.2) [a]	85.0 (78.9 - 91.1)	0.001

[*] Means within rows with a superscript letter in common did not differ significantly ($P \geq 0.05$)

[#] 2001 - 2004: Positive *P. aeruginosa* n = 58, Chronic *P. aeruginosa* infection n = 46

[^] 2005 - 2009: Positive *P. aeruginosa* n = 37, Chronic *P. aeruginosa* infection n = 29

[$] 2010 - 2014: Positive *P. aeruginosa* n = 43, Chronic *P. aeruginosa* infection n = 35

Table 2.6 Prevalence of infection among the 217 transitioning patients at the time of first review at The Prince Charles Hospital Adult Cystic Fibrosis Centre, n (%).

Years	Number of patients	*Pseudomonas aeruginosa*		*Staphylococcus aureus*		Achromobacter spp.	*S. maltophilia*	*B. cepacia* complex[^]	Aspergillus spp.	Scedosporium spp.	NTM[%]	No pathogen detected	No sample collected
		Positive	Chronic[$]	MSSA	MRSA								
2001-2004	76	58 (76.3)[a]	46 (60.5)	27 (35.5)	5 (6.6)	2 (2.6)	0 (0.0)	3 (3.9)	16 (21.1)	1 (1.3)	1 (1.3)	14 (18.4)	8 (10.5)
2005-2009	60	37 (61.7)[ac]	29 (48.3)	22 (36.7)	3 (5.0)	1 (1.7)	5 (8.3)[c]	0 (0.0)	16 (26.7)	5 (8.3)	2 (3.3)	16 (26.7)	16 (26.7)[c]
2010-2014	81	43 (53.1)[a]	35 (43.2)	21 (25.9)	2 (2.5)	5 (6.2)	6 (7.4)[c]	2 (2.5)	21 (25.9)	3 (3.7)	6 (7.4)	21 (25.9)	19 (23.5)[c]
Total	217	138 (63.6)	110 (50.7)	70 (32.3)	10 (4.6)	8 (3.7)	11 (5.1)	5 (2.3)	53 (24.4)	9 (4.1)	9 (4.1)	51 (23.5)	43 (19.8)
P value*		0.010	0.087	0.30	0.51	0.43	0.019	0.38	0.69	0.14	0.18	0.43	0.037

[a, b, c] numbers within columns with a letter in common are not significantly different

* Significant decrease in *P. aeruginosa* infection and a significant increase in *Stenotrophomonas maltophilia* infection. Rates of 'no sample collected' increased from the first study period 2001 - 2004.

MSSA: methicillin sensitive *Staphylococcus aureus*; MRSA: methicillin resistant *Staphylococcus aureus*; *S. maltophilia*: *Stenotrophomonas maltophilia*; NTM: nontuberculous mycobacteria.

[^] *Burkholderia cepacia* complex species: *B. cepacia* (n = 1), *B. cenocepacia* (n = 2), *B. multivorans* (n = 2), Indeterminant *B. cepacia* complex species (n = 1); one patient had more than one *B. cepacia* complex infection.

[%] Nontuberculous mycobacteria species: *M. abscessus* (n = 6), *M. avium* (n = 1), *M. chelonae* (n = 1), *M. intracellulare* (n = 2); two patients had more than one NTM infection.

[$] A modification of the Leed's criteria was used to define chronic *P. aeruginosa* infection: (i) in patients providing four or more sputum samples in a calendar year was determined when >50% of sputum samples were culture positive and (ii) in instances where fewer than four samples were collected or less than 50% of samples were culture positive; data from the previous year were reviewed and if the patients also fulfilled the above criteria they were also classified as having chronic infection.

2.4.5 Changing approach to therapy

Infants and young children in the study were given eradication therapy from 1999 and protocols aimed to eradicate the first and subsequent *P. aeruginosa* isolation of older children commenced at the Royal Children's Hospital (RCH) (Queensland) in 2004. For patients who were cared for at the RCH prior to transition to TPCH, microbiology results for this time were accessed (n = 189). Rates of *P. aeruginosa* infection in this subset were consistently high over all 3-yearly tertiles, however a 24% decrease in chronic infections was reported for the same time (Table 2.7). This change in practice was accompanied by a more intensive approach to regular sputum or upper airways sampling for microbiological culture.

Table 2.7 Changing rates of never, ever and chronic *Pseudomonas aeruginosa* infection in children transitioning from paediatric care [a].

Yearly Tertile	Total	Never Isolated *P. aeruginosa*	Ever Isolated *P. aeruginosa*	Chronic *P. aeruginosa* infection
2001 - 2004	63	1 (1.6)	62 (98.4)	46 (73.0)
2005 - 2009	54	8 (14.8)	46 (85.2)	29 (53.7)
2010 - 2014	72	5 (6.9)	67 (93.1)	35 (48.6)

[a] Children transitioning to TPCH from the RCH and excludes children transitioning from other Queensland and interstate hospitals (n = 28) as comprehensive (all of life) review of all microbiology culture results was not possible.

2.5 Discussion

Eradication therapy following *P. aeruginosa* acquisition is aimed at preventing the establishment of chronic infection in people with CF. This treatment is a widely recognised standard of paediatric CF care, is effective (i.e. culture negative) in up to 90% of patients and has been shown to extend the time to re-infection (i.e. next positive culture) (Doring *et al.* 2012; Langton Hewer and Smyth 2014; Mayer-Hamblett *et al.* 2015). In association with aggressive *P. aeruginosa* eradication protocols, and with a decreasing prevalence of *P. aeruginosa* infection, particularly in younger patients (Cystic Fibrosis Trust 2015; Cystic Fibrosis Trust 2006; Razvi *et al.* 2009; Salsgiver *et al.* 2015; Spicuzza *et al.* 2009), CF Patient Registries have demonstrated improving lung function and nutritional status, particularly over the past two decades (Cystic Fibrosis Foundation 2015). Such trends are less clear in adults (Lipuma 2010; Millar *et al.* 2009) and in particular rates of chronic infection remain uncertain due to the differences in definitions applied across studies (Pressler *et al.* 2011). In this study we sought to determine whether there has been a temporal change in the airway microbiology of adults with CF, with a specific focus on those transitioning from paediatric to adult CF care.

Our analysis demonstrates an overall decrease in the prevalence of total *P. aeruginosa* infection over 14 years in both the entire adult and transitioning patient cohorts. Additionally, these data demonstrated an overall decrease in prevalence of several other CF pathogens within the adult cohort. The decline in rates of *P. aeruginosa* infection is most likely attributable to an increase in the number of transitioning patients free of *P. aeruginosa* infection. Indeed, between 2001 and 2014, the frequency of *P. aeruginosa* (total and chronic) infection reduced by > 10%. The proportion of patients who could not provide a sputum sample also increased. Taken together, these data reflect the findings of other recent studies, indicating a healthier CF population at the time of transition from paediatric to adult care, in whom lower rates of *P. aeruginosa* infection are linked with improved clinical outcomes such as lung function and lung transplantation (Burgel *et al.* 2015; VanDevanter *et al.* 2008). Given the association of *P. aeruginosa* infection with accelerated lung function decline,

pulmonary exacerbations and increased mortality, successful eradication will remain an important component of CF care, however it may not be without risk. Increased exposure to antibiotics throughout life may lead to antibiotic allergy and toxicity, and the emergence of antibiotic-resistant airway pathogens (Bell and Reid 2014; Chmiel *et al.* 2014; Smith *et al.* 2016). Changing airway microbiology is presenting novel challenges for adult CF care, including how to best manage increasing centre size with cohorts of patients requiring different and increasingly complex approaches to infection control (Bell and Reid 2014). Furthermore, considering alternative approaches to microbiology surveillance in older patients including performing sputum induction may need to increasingly be adapted.

Here we observed an increase in the prevalence of *S. maltophilia* infection in younger patients at the time of transition to adult care that is consistent with other studies, although due to the small number of isolations reported this result needs to be interpreted with caution (Parkins and Floto 2015).

We have also shown a decreased prevalence of MSSA in the entire cohort over time that may reflect an ageing population. Although we demonstrated a decrease in the prevalence of *Aspergillus spp.* over time there were no major changes to laboratory practice or reporting that might obviously account for this, although again focus on intensive antibacterial treatments may contribute. The overall prevalence of MRSA also decreased throughout the study and the decrease in MRSA may also in part be related to the use of eradication protocols (Garske *et al.* 2004). Patients diagnosed with MRSA infection were managed according to strict infection control practices with eradication therapy established in 2001. The declining prevalence of MRSA in this patient population is exceptional particularly when compared to CF Centres in the USA where there has been a progressive increase in its prevalence over the past 15 years (Cystic Fibrosis Foundation 2015). Consistent with our earlier work, we demonstrated that fewer patients acquired *B. cepacia* complex infection in the latter part of the study (Ramsay *et al.* 2013; Zlosnik *et al.* 2015), which may reflect stringent infection control

practices and the use of antibiotic therapy to attempt to eradicate Burkholderia (Chmiel *et al.* 2014). Nevertheless, new cases of sporadic *B. cepacia* complex infection continue, emphasising the need for vigilant microbiological surveillance (Ramsay *et al.* 2013; Zlosnik *et al.* 2015).

A key finding of our study is the observation of declining prevalence of *P. aeruginosa* and improving pulmonary function of the transitioning patients. It is likely that aggressive strategies aimed at early identification by increased culture sampling and eradication of *P. aeruginosa* infection are important components of the improvements in lung function observed here. This study was not able to determine individual details of prior eradication therapy courses for all patients in the cohort. Rates of 'never' isolating *P. aeruginosa* infection have varied but were higher in the second and third periods of the study compared with the 2001 - 2004 period. 'Ever' *P. aeruginosa* isolation has been stable over time, yet rates with chronic *P. aeruginosa* infection have trended lower over time. The timing of such changes parallels with the changes in practice at the paediatric hospital, specifically the introduction of improved microbiological surveillance and antibiotic eradication therapy. Interestingly, transitioning patients with *P. aeruginosa* infection (total and chronic) demonstrated improved lung function which was not observed for those patients who did not have *P. aeruginosa* infection, which most likely reflects the relative preservation of lung function that we observed in patients without *P. aeruginosa* infection.

The definition of chronic *P. aeruginosa* infection had received considerable attention over the past 20 years and has been particularly important in the era of studies evaluating the effectiveness of *P. aeruginosa* eradication, yet the definition of chronic infection by the Leed's criteria has not been validated in the clinical care of adults with CF (Lee *et al.* 2003). Whilst, a relatively easy definition to apply, in routine clinical practice the provision of four or more samples in a year was only seen in ~50% of patients, even when those unable to provide any samples were excluded (median samples analysed over the 14-year study was 4). We modified the criteria and determined that $\geq$ 50% of

samples collected had *P. aeruginosa* cultured in a given year and if insufficient results were available, results from the prior year informed infection status and this identified many patients who fulfilled the criteria in the prior year but not in the subsequent year. Further studies are required to determine the value of this modification.

There are several limitations to this study. First, this cohort was a single adult centre study with most transitioning patients coming from local paediatric CF Centres. Importantly, whilst Registry data can provide information about the changing microbiological epidemiology over time, the depth of the information and its completeness may vary within Registries. Data presented in this study were obtained from a microbiology-specific database; as such we cannot comment on the relationship between *P. aeruginosa* prevalence and lung function or how treatments may be affecting the microbial diversity of the airways. Further, linkages with changing management approach (e.g. eradication therapy) are limited at this time point. Our study allows analysis of why such changing microbiology profiles may be occurring. Second, these analyses only included routine microbiology data generated by clinical laboratories and with the introduction of culture independent methods understanding the diversity of microorganisms in the CF airway has occurred, yet these advances had not at this point been incorporated into routine clinical practice. One significant change to microbiological identification which occurred during the study was the introduction of bioMerieux VITEK® MS. This methodology has been shown to accurately and rapidly identify a range of significant microbiological pathogens (Desai *et al.* 2012). Finally, unlike other studies which demonstrated a reduction respiratory pathogens, in particular *P. aeruginosa* infections, following the prescription of CFTR modulators (Heltshe *et al.* 2015; Hisert *et al.* 2017), due to the small number of patients within this cohort receiving ivacaftor treatment, this study was unable to determine if colonisation was affected.

In conclusion, we demonstrate the prevalence of *P. aeruginosa*, and several other common respiratory pathogens has decreased over the past 14 years in this adult CF cohort, despite no change to eradication techniques or hospital management protocols during that time. Of particular note we observed that lung function has improved in transitioning patients with *P. aeruginosa* infection. Furthermore, we have adapted the definition of chronic infection to better suit a clinical setting, and by doing so demonstrate that prevalence of chronic *P. aeruginosa* remains unchanged in this adult centre, despite a decline in total *P. aeruginosa* infection prevalence. Amongst many challenges, how best to manage the growing number of healthier patients who have fewer respiratory infections will continue in adult CF centres.

Chapter 3: Longitudinal analysis of *Pseudomonas aeruginosa* genotypes

3.1 Abstract

Background: Shared strains of *Pseudomonas aeruginosa* have been isolated from patients with cystic fibrosis (CF) in many countries worldwide. Respiratory infection caused by these strains has been associated with poor health outcomes for patients. Due to the lack of an environmental reservoir, patient to patient transmission is the most likely source of acquisition. Consequently, cohort segregation has been vital in reducing and preventing acquisition of these shared strains. Currently limited work has been conducted to assess strain diversity without the implementation of such an intervention. Therefore, through the use of longitudinally collected *P. aeruginosa* isolates, this study will assess the change in strain diversity, incidence and clinical impact of shared strains prior to the implementation of patient segregation.

Methods: Sputum samples from patients with CF attending The Prince Charles Hospital, Australia were collected at two cross sectional time points (Baseline and Final) and screened for *P. aeruginosa*. Isolates were genotyped (SNP-based assay) and strain diversity at both time points determined. In addition, clinical measures, including age, sex, CFTR genotype, co-morbidities, hospitalisation, lung function and nutritional status were collected.

Results: The prevalence of the AUST-02 strain decreased (P = 0.020) the prevalence of the AUST-06 strain increased (P = 0.001) during this study. Following adjustment for clinical characteristics patients with the AUST-02 strain were > 3 times more likely to die or require lung transplantation. This result was reflected in the high number of patients harbouring this strain that did die or required lung transplantation during this study (20%). Despite no change to clinical characteristics over time for the entire cohort, patients who acquired a shared strain, in particular AUST-06, reported the largest decline to health over the study period.

Conclusion: This study confirms that high rates of strain diversity is present within this cohort and despite the change in the prevalence of specific shared strains, overall no change in the rates of shared strains were noted. Similar to previous reports, patients harbouring a shared strain infection have a greater requirement for hospital-based care and a greater decline in lung function compared to patients with no shared strain infection'. However, further characterisation, yet to be undertaken, of AUST-02 virulence using animal and other experimental models is needed to better understand the impact this infection has on the health of patients.

3.2 Introduction

Cystic fibrosis (CF) is the most common autosomal recessive disorder affecting Caucasians. The leading cause of mortality and morbidity among people with CF is chronic suppurative lung disease, often a result of respiratory infection caused by the bacterium *Pseudomonas aeruginosa* (Ratjen *et al.* 2015). Acquisition of *P. aeruginosa* from environmental sources has historically been considered the primary route of acquisition; however, person-to-person transmission and nosocomial acquisition have also been identified as potential infection sources (Jones *et al.* 2003; Kidd *et al.* 2013; Kidd *et al.* 2015; Knibbs *et al.* 2014; Ranganathan *et al.* 2013; Saiman *et al.* 2014; Wiehlmann *et al.* 2012). To reduce the risk of cross-infection infection control guidelines recommend the implementation of numerous practices including hand hygiene, rigorous cleaning of equipment and facilities designated areas for airway clearance and nebuliser use and single rooms for hospital admissions (Saiman *et al.* 2014). Improved infection control has been shown to reduce the incidence and prevalence of *P. aeruginosa* infection, as well as, the onset of chronic infection in several settings (Frederiksen *et al.* 1999; Wiehlmann *et al.* 2012).

Genotyping of *P. aeruginosa* isolates has shown that unrelated patients can harbour genetically indistinguishable (shared) strains which have been associated with increased treatment burden and poorer health outcomes compared to patients infected with unrelated (non-shared) strains (Aaron *et al.* 2010; Armstrong *et al.* 2002; Cheng *et al.* 1996; Kidd *et al.* 2013; O'Carroll *et al.* 2004; Scott and Pitt 2004; van Mansfeld *et al.* 2009). Cohort segregation based on shared strain infection status has demonstrated that separation of patients, limiting interactions during all hospital attendances and also preventing social contact, can have a substantial impact on the incidence and prevalence of shared strain infections (Ashish *et al.* 2013; Griffiths *et al.* 2005). However, not all studies have reported a significant change to rates of *P. aeruginosa* acquisition based on these interventions. van Mansfeld and colleagues reported that although no new acquisitions of a predominant Dutch shared strain were identified following the introduction of strict segregation, overall there was no change in the incidence

of chronic infection (van Mansfeld *et al.* 2016). Conversely, the implementation of patient segregation has not been shown to significantly affect the rates of non-shared strain infections (Ashish *et al.* 2013; Griffiths *et al.* 2005; Jones *et al.* 2005), and furthermore, the strict adherence to infection control and hygiene practices has increased the heterogeneity of strains, suggesting environmental acquisition is still occurring (Griffiths *et al.* 2005; Kidd *et al.* 2013; Matt *et al.* 2014).

Despite evidence of reduced shared strain infection supporting cohort segregation not all CF centres have the facilities, resources and infrastructure to genotype isolates on a regular basis and provide single room inpatient accommodation for all patients. Over the past ten to fifteen years, there has been a gradual update to more stringent cohort segregation as hospitals and CF centres have adapted resources and facilities to ensure that such care to be implemented. In a prospective observational study conducted by Aaron and colleagues, although they reported a high prevalence of shared strains, there were relatively few new acquisitions observed during the study. It is also unclear if the prevalence rates of shared strain infections within this cohort changed over time (Aaron *et al.* 2010). Within Australia, cross-sectional analyses have identified a number of prevalent shared strains and demonstrated a decline in these strains following implementation of cohort segregation(Bradbury *et al.* 2008; Griffiths *et al.* 2005; Kidd *et al.* 2013; O'Carroll *et al.* 2004; Tingpej *et al.* 2010). To date longitudinal analyses of changing prevalence of shared strains have been limited.

Therefore, the aim of this prospective longitudinal study was to undertake a seven year study in which the prevalence and incidence of predominant shared stains was evaluated within the adult CF population at The Prince Charles Hospital (TPCH), Brisbane, Australia. A secondary aim of this work was to determine if acquisition of a shared strain was associated with worse clinical outcomes.

3.3 Materials and Methods

3.3.1 Study Population

This study comprised of patients recruited into a national cross-sectional study (i.e. the Australian Clonal Pseudomonas in CF [ACPinCF] Study) aimed at assessing the prevalence and clinical impact of shared *P. aeruginosa* strain infections within the Australian CF population (Kidd *et al.* 2013). During the ACPinCF Study patients provided expectorated sputum samples at recruitment and annually thereafter for up to five years. Briefly, *P. aeruginosa* (three colonies per sample) isolated from culture positive samples were transferred to a research lab for confirmatory molecular identification and genotyping.

The current investigation was focused on one of the largest adult ACPinCF Study centres (TPCH, Brisbane, Queensland; ACPinCF study identifier QLD02 (Kidd *et al.* 2013) and assessed the cross-sectional prevalence of the three predominant shared strains, AUST-01, AUST-02 and AUST-06 (point prevalence ≥ 10%) at recruitment (Baseline; 2007 – 2009) and then after approximately five years (Final; 2012 – 2014) or study censor point (i.e. death, lung transplantation, patient relocation or no further *P. aeruginosa* isolated). For inclusion into this study, *P. aeruginosa* positive sputum samples had to be collected from patients at least one sampling time points. Comparison of each patient's *P. aeruginosa* genotyping results at the two time points was undertaken to identify potential incidence cases of shared strain infection. For patients identified as an incidence case, genotyping of annually collected isolates was performed. Clinical characteristics, including age, sex, CFTR genotype, co-morbidities, number of days in hospital and number of hospital admissions were collected at Baseline and Final sample collection. Lung function (i.e. forced expiratory volume in 1 second [FEV_1]) and nutrition (body mass index [BMI]; kg/m^2) data recorded at each hospital visit across the study period was collected for all participants (Hankinson *et al.* 1999; Wang *et al.* 1993). Baseline clinical data and bacterial isolates collected from ACPinCF Study participants that were recruited to other study centres and transferred to TPCH during the study period were also included in the analysis.

3.3.2 Sample collection and isolate identification

Sputum *P. aeruginosa* isolates were cultured and identified by diagnostic microbiology laboratories using standard techniques (Kidd *et al.* 2009). When *P. aeruginosa* was identified, three morphologically distinct colonies (where possible) were selected for inclusion in this study and forwarded onto the research laboratory for storage at -80 °C until required for further testing (Kidd *et al.* 2013).

3.3.3 Isolate storage and retrieval

On arrival in the research laboratory each isolate was sub-cultured onto nutrient agar to assess purity and then stored at -80 °C in 15% glycerol (Kidd *et al.* 2009). When required for testing, each stored isolate was cultured overnight on LB agar at 37 °C and then sub-cultured using the same conditions prior to analysis (Syrmis *et al.* 2013).

3.3.4 DNA Extraction

Heat denatured bacterial DNA was prepared as follows. A 1.0 McFarland suspension was prepared from a 24-hour single-colony subculture in 2.0 mL of water and heated for 20 minutes at 95 °C. Each suspension was then centrifuged at 14,000 rpm for 4 minutes, with the supernatant stored at -80 °C prior to genotyping (Anuj *et al.* 2009; Syrmis *et al.* 2013).

3.3.5 Confirmatory Identification

All isolates underwent *P. aeruginosa*-specific real time duplex PCR assay targeting the *ecfX* and *gyrB* genes to confirm their identity (Anuj *et al.* 2009). Each PCR reaction mix consisted of 12.5 µL of Qiagen Quantitect Probe Master Mix, 10 pmol/µL each forward and reverse primer (Table 3.1), 20 pmol/µL of each TaqMan probe (Table 3.2) and 2 µL of DNA template, made up to a final reaction volume of 25 µL with DNA-free water. Thermal cycling conditions comprised a single initial cycle at 95 °C for 15 minutes, followed by 50 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds and

one final cooling cycle of 40 °C for 30 seconds. Cycling and amplicon detection was performed on a LightCycler®480 instrument. The fluorescent probes for *ecfX* and *gyrB* were labelled with Yakima Yellow (YAK) and a fluorescein, FAM, so they could be detected in the JOE (523 - 568 nm) and FAM (483 - 533 nm) channels, respectively (Anuj *et al.* 2009; Kidd *et al.* 2009). Control strain isolates including *Achromobacter xylosoxidans* LMG 1863 and representative AUST-01 and AUST-02 *P. aeruginosa* isolates were included in each assay.

Table 3.1 Primers used in *P. aeruginosa* specific real time duplex PCR assay.

Name	Primer Sequence 5'-3'
ecfX-fwd	CGCATGCCTATCAGGCGTT
ecfX-rvs	GAACTGCCCAGGTGCTTGC
gyrB-fwd	CCTGACCATCCGTCGCCACAAC
gyrB-rvs	CGCAGCAGGATGCCGACGCC

Table 3.2 Probes used in *P. aeruginosa* specific real time duplex PCR assay.

Name	Probe Sequence 5'-3'
ecfX-probe	JOE-ATGGCGATTTGCTGCGCTTCCT-BHQ1
gyrB-probe	FAM-CCGTGGTGGTAGACCTGTTCCCAGACC-BHQ1

3.3.6 20-SNP iPLEXMassARRAY

A combination of 20 informative single nucleotide polymorphisms (SNPs) capable of differentiating the dominant shared strains were identified from sequence data found on the *P. aeruginosa* PubMLST website (Jolley and Maiden 2010; *Pseudomonas aeruginosa* PubMLST Database 2012). As described by Syrmis and colleagues, 24 amplification and 21 extension primers, have been designed to identify the 20 most informative SNPs (Syrmis *et al.* 2014; Syrmis *et al.* 2013). All PCRs described below were prepared and performed by AGRF using the Agena Bioscience, Inc. iPLEX® assay protocols and analysed on the MassARRAY® System and using TyperAnalyzer software.

3.3.7 DNA Amplification

Each reaction contained 1 x PCR buffer (supplemented with 2 mM MgCl2), 2 mM MgCl2, 500 µM dNTP mix, 100 nM of each amplification primer, (Syrmis *et al.* 2014; Syrmis *et al.* 2013) 1 U/rxn PCR enzyme and 2 µL of DNA template, adjusted with water to a final volume of 5 µL. Thermal cycling run conditions for the amplification PCR were, an initial cycle of 95 °C for 2 minutes, followed by 45 cycles of 95 °C for 30 seconds, 56 °C for 30 seconds and 72 °C for 60 seconds with a final extension cycle at 72 °C for 5 minutes. SAP treatment, to dephosphorylate unincorporated dNTP's, was then performed to prepare the template for SNP analysis. The SAP reaction mixed consisted of 1X SAP buffer and 0.5 U/µL SAP enzyme, adjusted with water to a final volume of 7 µL. An initial incubation of 37 °C for 40 minutes and enzyme deactivation of 85 °C for 5 minutes constituted the thermal cycling conditions (Syrmis *et al.* 2013).

3.3.8 Extension PCR

A prepared amplification PCR template iPLEX (Sequenom) reaction mix was added to each well. This extension mix contained 0.2 µL iPLEX Buffer Plus, 0.2 µL iPLEX Termination Mix, 0.94 µL extension primers and 0.04 µL iPLEX enzyme adjusted with water to a final volume of 2µL. Thermal cycling conditions consisted of an initial cycle of 95 °C for 30 seconds, followed by 40 cycles of 95 °C for 5 seconds, with five subcycles of 52 °C for 5 seconds and 80 °C for 5 seconds, completed with one cycle of 72 °C for 3 minutes (Syrmis *et al.* 2013).

3.3.9 Definitions

Shared Strains: Comprise AUST-01, AUST-02 and AUST-06 which represented the most prevalent (point prevalence of ≥ 10%) *P. aeruginosa* strains isolated within TPCH Adult CF centre between 2007 and 2009 (Kidd *et al.* 2013).

Minor Shared Strains: Refer to strains which have been identified in two or more of the participants of this current study with a point prevalence of < 10%. In some analyses (unique and minor shared strain) are referred to as 'non-shared strains'.

Unique Strains: Refer to singleton strains which have only been isolated in one patient throughout the duration of the study (Kidd *et al.* 2013).

Hospital Admissions: The number of hospital admissions resulting from respiratory symptoms occurring during the calendar year in which Baseline and Final sample collections were obtained.
Hospital Days: The number of inpatients days, resulting from respiratory symptoms which occurred during the calendar year in which Baseline and Final sample collections were obtained.

3.3.10 Statistical Analysis

For continuous variables summary statistics are presented as either mean (standard deviation, SD) or median (interquartile range, IQR) depending on the distribution. For categorical variables summary statistics are presented as frequency (percentage). Differences between groups at baseline were investigated using linear regression with 'group' included as the main effect. Differences across time were investigated using linear regression with time included as the main effect.

3.4 Results

3.4.1 Study Population

Figure 3.1 shows a total of 266 patients provided one or more *P. aeruginosa*-positive sample during the study period. One hundred and seventy-one patients were recruited and managed within TPCH Adult CF Centre (ACFC), TPCH at Baseline (2007 – 2009), and 222 patients provided a Final *P. aeruginosa*-positive sample between 2012 and 2014. After Baseline recruitment, 67 patients transferred into TPCH from other CF centres, 28 existing patients produced *P. aeruginosa*-positive sputum cultures and 44 patients were censored prior to study completion (death n = 8, lung transplantation n = 21, relocation n = 14, and no *P. aeruginosa* isolated n = 1). Of the 171 patients recruited to TPCH at Baseline, 127 (74.3%) also provided a *P. aeruginosa*-positive sputum at the Final sample collection time point.

The overall mean duration between Baseline and Final sample collection for the entire patient cohort was 4.3 years (SD ± 1.6 years) and the mean annualised rate of lung function decline for all patients was -1.3 FEV_1 % predicted. With the exception of age, there were no differences in the clinical characteristics of the patients that provided samples at the Baseline (n = 171) and Final (n = 222) sample collection time points (Table 3.3A). In contrast, the 127 patients who attended the TPCH throughout the study experienced an increased number of hospital admissions ($P = 0.034$) and in- patient days ($P = 0.009$) and a reduction in lung function ($P = 0.002$) at the latter sample collection time point (Table 3.3B).

Figure 3.1 Flowchart of patient recruitment and censoring from Baseline (2007-2009) and Final (2012-2014) sample collection at The Prince Charles Hospital Adult CF Centre, (n = 266).

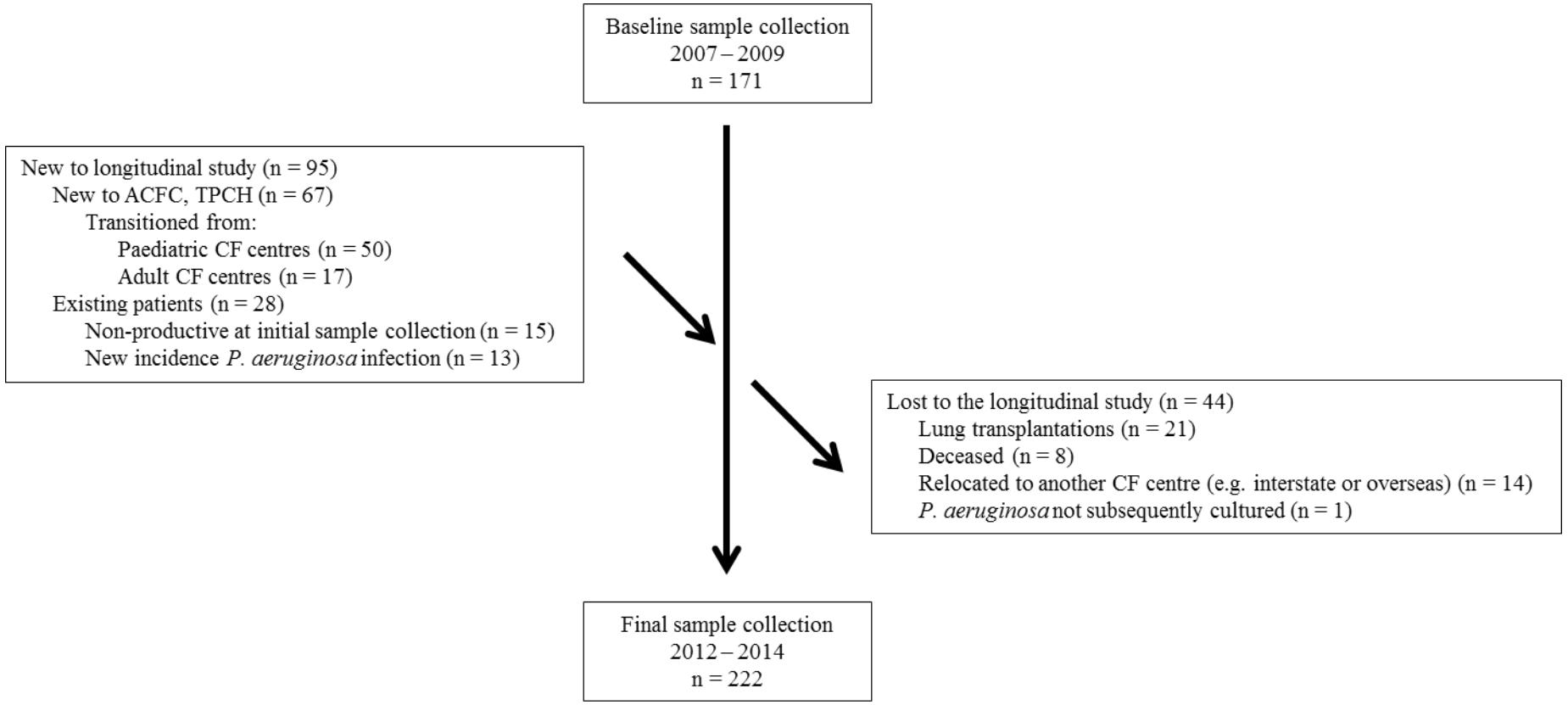


Table 3.3A Clinical characteristics of study participants attending The Prince Charles Hospital Adult CF Centre across the Baseline (n = 171) and Final (n = 222) sample collection time points.

	Baseline	Final	*P*-value
Subjects, n	171	222	
Age at collection, years, mean (95% CI)	28.4 (27.1 - 29.7)	30.2 (29.0 - 31.5)	
Sex, males, n (%)	102 (59.6)	124 (55.9)	0.451
CFTR, n (%)			
Minimal function	135 (78.9)	176 (79.3)	
Residual function	12 (7.0)	13 (5.9)	0.920
Not classified	16 (9.4)	24 (10.8)	
Not available	8 (4.7)	9 (4.1)	
Pancreatic Insufficient, n (%)	159 (93.0)	206 (92.9)	0.942
Insulin Dependent Diabetes, n (%)	30 (17.5)	38 (17.1)	0.934
FEV_1 % predicted, mean (95% CI)	59.8 (56.4 - 63.1)	60.8 (57.7 - 63.9)[a]	0.655
Mean (95% CI) Annual FEV_1 % predicted decline	-1.3 (-1.4 to -1.2)		
BMI, mean (95% CI)	22.0 (21.5 - 22.6)	22.5 (22.0 - 23.0)[a]	0.246
Hospital Admissions - Respiratory, median (IQR)	1.0 (0.0 - 2.0)	1.0 (0.0 - 3.0)	0.319
Hospital Days - Respiratory, median (IQR)	11.0 (0.0 - 23.0)	13.0 (0.0 - 33.0)	0.088

[a] FEV_1 % predicted and BMI data were not available for five and two patients, respectively.

Table 3.3B Clinical characteristics of study participants attending The Prince Charles Hospital Adult CF Centre throughout the study (n = 127).

	Baseline	Final	*P*-value
Age at collection, years, mean (95% CI)	27.9 (26.5 - 29.2)	33.0 (31.6 - 34.3)	-
Sex, male n (%)	83 (65.4)		-
CFTR, n (%)			
Minimal function	108 (85.0)		
Residual function	10 (7.9)		-
Not classified	8 (6.3)		
Not available	1 (0.8)		
Pancreatic Insufficient, n (%)	119 (93.7)	119 (93.7)	1.000
Insulin Dependent Diabetes, n (%)	16 (12.6)	22 (17.3)	0.572
FEV_1 % predicted, mean (95% CI)	64.7 (61.2 - 68.1)	56.8 (53.1 - 60.5)[a]	0.002
Mean (95% CI) Annual FEV_1 % predicted decline	-1.3 (-1.4 to -1.2)		
BMI, mean (95% CI)	22.4 (21.8 - 23.1)	23.2 (22.5 - 23.8)[a]	0.127
Hospital Admissions - Respiratory, median (IQR)	1.0 (0.0 - 2.0)	1.0 (0.0 - 3.0)	0.034
Hospital Days - Respiratory, median (IQR)	10.0 (0.0 - 22.0)	14.0 (0.0 - 34.0)	0.009

[a] FEV_1 % predicted and BMI data were not available for two and one patients, respectively.

3.4.2 Strain diversity

A total of 509 and 658 isolates were available for genotyping at the Baseline and Final collection time points, respectively. Of the 246 patients for which isolates were available for genotyping at both time points, 159 (65%) showed the same *P. aeruginosa* strain(s) at Baseline and Final sample collection. Of these patients, shared strain retention was noted for 81 patients (51%).

Over the study period a significant decrease in the prevalence of the AUST-02 shared strain, (Baseline: 37.4%, Final: 26.6%; $P = 0.020$) and an increase in the prevalence of the AUST-06 shared strain (Baseline: 11.1%, Final: 24.8%; $P = 0.001$) was observed. In contrast, the prevalence of AUST-01 (Baseline: 12.3%, Final: 11.3%; $P = 0.760$), minor shared (Baseline: 33.9%, Final: 32.9%; $P = 0.830$) and unique strain (Baseline: 20.5%, Final: 22.5%; $P = 0.620$) infections remained similar (Table 3.4).

Table 3.4 Strain diversity of *Pseudomonas aeruginosa* isolates cultured from study participants attending The Prince Charles Hospital Adult CF Centre across the Baseline (n = 171) and Final (n = 222) sample collection time points.

P. aeruginosa strain	Baseline n = 171 n (%)	Final n = 222 n (%)	Percentage Mean Difference (95% CI)	*P*-value
AUST-01	21 (12.3)	25 (11.3)	-1.0 (-7.5 - 5.4)	0.760
AUST-02	64 (37.4)	59 (26.6)	-10.9 (-20.1 - 1.6)	0.020
AUST-06	19 (11.1)	55 (24.8)	13.7 (6.2 - 21.0)	0.001
Minor Shared strains	58 (33.9)	73 (32.9)	-1.0 (-10.4 - 8.4)	0.830
Unique strains	35 (20.5)	50 (22.5)	2.1 (-6.1 - 10.2)	0.620

3.4.3 Clinical characteristics of patients stratified according to strain type infection

The clinical characteristics of patients attending TPCH for the complete duration of the study (n = 127) stratified according to strain type infection at Baseline and Final sample collection time points are presented in Table 3.5. Despite the decline in prevalence over time, patients harbouring the AUST-02 strain remained the largest proportion of this cohort. Patients with AUST-01, AUST-02 and AUST-06 were younger at Baseline sample collection compared with those harbouring minor shared and unique strains. Males comprised almost all the patients in the AUST-01 strain group; whereas the gender distribution for all other strain groups was more reflective of the entire cohort. Rates of co-morbidities were similar across each of the strain groups.

The association between strain and clinically relevant parameters was explored. This analysis revealed that compared to the AUST-02 strain group, patients with AUST-06 infection showed significantly poorer lung function (FEV_1% predicted: 46.4% versus 59.8% [P = 0.011]) at Baseline sample collection. Mean FEV_1% predicted of AUST-06 infected patients at Baseline was also substantially lower than those showing AUST-01 (55.6%), minor shared (59.6%) and unique strain (54.8%) infection; though, these differences were not statistically significant. At Baseline, patients with AUST-02 and minor strain infections also experienced less hospital admissions (Minor, P = 0.007) and fewer inpatient days (AUST-02, P = 0.036, Minor, P = 0.012) compared to patients in the AUST-06 strain group. When assessing the deterioration in clinical markers across the study period no differences in the annual decline of lung function or rates of hospitalisation at the Final sample collection time point across the different strain groupings was observed. However, this analysis did indicate a trend towards greater FEV_1 % predicted decline for patients with AUST-06 infection. In addition, all patient groups, particularly those with AUST-06, showed a significantly increased annual decline in nutritional status compared to patients with AUST-01 infection.

Table 3.5 Clinical characteristics of subjects treated within at TPCH ACFC throughout the study, stratified according to *P. aeruginosa* genotype at the baseline and final collection time points.

	AUST-01		AUST-02		AUST-06		Minor		Unique	
	Baseline	Final	Baseline	Final	Baseline	Final	Baseline	Final	Baseline	Final
Subjects, n (%)	15 (11.8)	14 (11.0)	46 (36.2)	42 (33.1)	15 (11.8)	31 (24.4)	44 (34.7)	42 (33.1)	24 (18.9)	24 (18.9)
Age at collection, years, mean (95% CI)	26.4 (22.7 - 30.1)	31.2 (27.3 - 35.0)	25.7 (23.8 - 27.5)	30.7 (28.8 - 32.6)	26.2 (22.9 - 29.6)	33.5(30.8 - 36.3)	29.3 (26.9 - 31.6)	35.0 (32.5 - 37.5)	31.7 (27.5 - 35.9)	35.3 (31.1 - 39.4)
Sex, males, n (%)	14 (93.3)	14 (100.0)	31 (67.4)	29 (69.1)	8 (53.3)	19 (61.3)	27 (61.4)	25 (59.5)	14 (58.3)	14 (58.3)
CFTR, n (%)										
Minimal function	13 (86.7)	12 (85.7)	41 (89.1)	38 (90.5)	11 (73.3)	27 (87.1)	38 (86.4)	34 (81.0)	20 (83.3)	21 (87.5)
Residual function	2 (13.3)	2 (14.3)	3 (6.5)	2 (4.8)	3 (20.0)	3 (9.7)	1 (2.3)	2 (4.8)	3 (12.5)	2 (8.3)
Not classified	0 (0.0)	0 (0.0)	2 (4.4)	2 (4.8)	0 (0.0)	0 (0.0)	5 (11.4)	6 (14.3)	1 (4.2)	1 (4.2)
Not available	0 (0.0)	0 (0.0)		0 (0.0)	1 (6.7)	1 (3.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pancreatic Insufficient, n (%)	15 (100.0)	14 (100.0)	45 (97.8)	42 (100.0)	14 (93.3)	29 (93.6)	40 (90.9)	38 (90.5)	21 (87.5)	22 (91.7)
Insulin Dependent Diabetes, n (%)	0 (0.0)	0 (0.0)	6 (13.0)	7 (16.7)	3 (20.0)	9 (29.0)	8 (18.2)	8 (19.1)	3 (12.5)	5 (20.8)
FEV_1 % predicted, mean (95% CI) [a]	55.6 (45.4 - 65.8)	52.4 (39.8 - 64.9)	59.8 (54.3 - 65.3) *	54.2 (47.7 - 60.7)	46.4 (38.4 - 54.4)	44.1 (37.4 - 50.9)	59.6 (53.3 - 65.9)	54.5 (47.8 - 61.3)	54.8 (48.3 - 61.3)	46.8 (37.5 - 56.1)
Mean (95% CI) Annual FEV1% predicted decline	-1.3 (-2.4 to -0.2)		-1.6 (-2.3 to -0.9)		-2.3 (-3.8 to -0.9)		-1.3 (-2.0 to -0.7)		-1.1 (-2.0 to -0.2)	
BMI, mean (95% CI) [b]	22.4 (20.3 - 24.4)	25.0 (23.0 - 27.0)	22.3 (21.3 - 23.3)	22.3 (21.1 - 23.4)	21.8 (20.3 - 23.3)	21.9 (20.6 - 23.3)	22.7 (21.6 - 23.8)	23.8 (22.3 - 25.4)	22.3 (20.4 - 24.1)	21.9 (20.3 - 23.5)
Mean (95% CI) Annual BMI decline	0.47 (0.29 to 0.66)		0.13 (0.01 to 0.24) ^		-0.02 (-0.28 to 0.23) ^		0.08 (-0.03 to 0.20) ^		-0.01 (-0.17 to 0.14) ^	
Hospital Admissions - Respiratory, median (IQR)	1 (1 - 2)	1 (0 - 2)	1 (0 - 2) *	1 (1 - 3)	2 (0 - 4)	3 (2 - 4)	1 (0 - 1) *	1 (0 - 2)	1 (0 - 2)	2 (0.5 - 3)
Hospital Days - Respiratory, median (IQR)	11 (7 - 14)	14 (0 - 20)	10.5 (0 - 24)	13 (8 - 36)	12 (0 - 51)	42 (17 - 63)	8 (0 - 13) *	11.5 (0 - 20)	7 (0 - 22.5)	14 (4.5 - 32.5)

[a] FEV1 % predicted results unavailable from between 1 and 8 patients

[b] BMI results unavailable for between 1 and 9 patients

* Compared to AUST-06 Baseline results these results were statistically different ($P \leq 0.05$)

^ Compared to the mean annual decline of AUST-01 these results were statistically different ($P \leq 0.05$)

3.4.4 Clinical characteristics of patients with a stable *P. aeruginosa* genotype infection

Of the 159 patients who retained the same *P. aeruginosa* genotype(s) throughout the study period, non-shared and shared strains were reported in 73 and 86 of subjects, respectively. Fifty-one (32%) of these patients attended a centre other than TPCH at Baseline, while 78 (49%) attended TPCH throughout the entire study period. The remaining 30 patients provided *P. aeruginosa* sample for genotyping up until the point of censoring and did not contribute Final samples due to lung transplantation (n = 11), death (n = 5), relocation to another CF centre (n =11), lack of sputum productivity (n = 1).

Table 3.6 shows that compared to patients with stable shared infection, those with stable non-shared strain infections were older and more likely to be female, but showed similar lung function and nutritional parameters. Furthermore, decline in lung function over time did not differ between the two groups (P = 0.133). Importantly, there was a significant difference in hospitalisation rates between the two groups at Baseline. Patients with a non-shared strain infection had on average one admission fewer than those with a shared strain (P = 0.010) and 14 fewer days in hospital (P = 0.001) per year; however, when this was compared at study completion there was no difference (Admissions P = 0.354, Days P = 0.096) (Table 6).

Table 3.6 Comparison of clinical characteristics of the study participants with a stable infection at Baseline and Final sample collection time points.

	Same Non-Shared Strain at both collection time points		Same Shared Strain at both collection time points	
	Baseline	Final	Baseline	Final
	n = 73		n = 86	
Age at collection, years, mean (95% CI)	28.4 (25.8 - 31.0)	32.4 (30.0 - 35.0)	24.1 (22.6 - 25.6)	28.6 (27.1 - 30.1)
Sex, males, n (%)	28 (38.4)		56 (65.1)	
Lung Transplantation, n (%)	4 (5.5)		9 (10.5)	
Deceased, n (%)	3 (4.1)		2 (2.3)	
Pancreatic Insufficient, n (%)	63 (86.3)	65 (89.0)	84 (97.7)	84 (97.7)
Insulin Dependent Diabetes, n (%)	9 (12.3)	13 (17.8)	12 (14.0)	15 (17.4)
FEV_1 % predicted, mean (95% CI)	61.1 (58.0 - 66.3) [a]	58.6 (53.1 - 63.9)	66.1 (61.1 - 71.2)	59.4 (53.8 - 65.0) [c]
BMI, mean (95% CI)	21.6 (20.7 - 22.4) [a]	22.0 (21.2 - 22.9) [b]	21.6 (20.8 - 22.3)	22.4 (21.6 - 23.3) [c]
Hospital Admissions - Respiratory, median (IQR)	1.0 (0.0 - 1.0) *	1.0 (0.0 - 2.0)	1.0 (1.0 - 3.0) *	1.0 (0.0 - 3.0)
Hospital Days - Respiratory, median (IQR)	3.0 (0.0 - 14.0) *	12.0 (0.0 - 26.0)	13.0 (3.0 - 28.0) *	15.0 (0.0 - 36.0)

Clinical parameters were unavailable for a number of patients at both time points; therefore total numbers tested [a] n = 71, [b] n = 69, [c] n = 80.

* Denotes a statistical difference between Baseline results ($P = \leq 0.05$)

FEV_1 % predicted decline was not calculated as 51 patients had care at another CF for the Baseline sample collection.

3.4.5 Clinical characteristics of patients who acquired a new shared strain infection

Thirty-four patients acquired a new shared strain of *P. aeruginosa* during the study. As there was only one patient who acquired AUST-01 and one patient that acquired AUST-02 and AUST-06 combined, they were excluded from further analysis. Of the 32 incident cases analysed, eight (25%) patients acquired AUST-02, while 24 (75%) acquired AUST-06. Of the 24 patients that acquired AUST-06, 16 (67%) were managed at TPCH ACFC throughout the entire study period. Therefore, the increased prevalence of the AUST-06 strain could not be explained by new cases coming into TPCH ACFC from other healthcare settings.

The clinical characteristics of patients with an existing AUST-02 infection and those who acquired this strain during the study are presented in Table 3.7. This analyses shows that these patient groups were similar for age, gender distribution, CFTR genotype and co-morbidities. Patients who acquired AUST-02 appeared to have greater lung function decline (Retention: -1.8 FEV_1 % predicted, Acquisition: -4.0 FEV_1 % predicted; $P = 0.894$), though this was not significant. Nutritional status ($P = 0.629$) and hospitalisation requirements (Admissions, $P = 0.671$; Days, $P = 0.878$) were similar between the groups. Interestingly, a significant increase in the number of hospital days at Baseline compared to Final sample collection was observed for the patients who acquired an AUST-02 infection (Baseline n = 4, Final n = 11.5; $P = 0.04$), whereas there was no change to hospital days reported for the patients who retained an AUST-02 infection (Baseline n = 12, Final n = 13.5; $P = 0.51$).

The clinical characteristics of patients with an existing AUST-06 infection are compared with those who acquired this strain during the study in Table 3.8. This analyses shows patients who acquired the AUST-06 strain were older and that the proportion of patients with minimal CFTR function and co-morbidities were similar between the two patient groups. The lung function values reported at Baseline were considerably different between the groups, this however did not achieve significance

(P = 0.115). Comparison of Baseline and Final clinical data showed that patients who acquired AUST-06 had similar lung function decline (Retention: -2.6 FEV_1 % predicted, Acquisition: -2.8 FEV_1 % predicted; P = 0.121), hospitalisation rates (Admissions, P = 0.676; Days, P = 0.907) and better nutrition that those who retained this strain over time (P = 0.015).

Table 3.7 Clinical characteristics of patients with a recent AUST-02 acquisition compared to those who retained an AUST-02 infection for the duration of the study.

	Retention of AUST-02 strain n = 36		Acquisition of AUST-02 strain n = 6	
	Baseline	Final	Baseline	Final
Average age at collection, years, mean (95% CI)	25.4 (23.3 - 27.4)	30.6 (28.5 - 32.6)	26.6 (17.9 - 35.2)	31.4 (23.1 - 39.7)
Sex, males, n (%)	25 (69.4)		4 (66.7)	
CFTR, n (%)				
Minimal function	33 (91.7)		5 (83.3)	
Residual function	2 (5.6)		0 (0.0)	
Not classified	1 (2.8)		1 (16.7)	
Not available	0 (0.0)		0 (0.0)	
Lung Transplantation, n (%)	1 (2.8)		1 (16.7)	
Deceased, n (%)	2 (5.6)		2 (33.3)	
Pancreatic Insufficient, n (%)	36 (100.0)	36 (100.0)	6 (100.0)	6 (100.0)
Insulin Dependent Diabetes, n (%)	4 (11.1)	6 (16.7)	1 (16.7)	1 (16.7)
FEV_1 % predicted, mean (95% CI)	70.6 (64.8 - 76.4)	61.3 (54.0 - 68.6) [a]	66.5 (43.4 - 89.6)	48.8 (31.2 - 66.4)
Mean (95% CI) Annual FEV_1% predicted decline	-1.8 (-2.5 to -1.1) [b]		-4.0 (-9.8 to 1.8)	
BMI, mean (95% CI)	22.1 (21.0 - 23.2)	22.6 (21.5 - 23.8)	20.2 (16.6 - 23.7)	19.6 (16.3 - 22.9)
Mean (95% CI) Annual BMI decline	0.1 (-0.3 to 0.2)		-0.1 (-0.6 to 0.3)	
Hospital Admissions - Respiratory, median (IQR)	1.5 (1.0 - 2.0)	1 (1.0- 3.0)	1.0 (0.0 - 4.0)	1.0 (0.0 - 2.0)
Hospital Days - Respiratory, median (IQR)	12.0 (3.0 - 23.0)	13.5 (9.0 - 36.0)	4.0 (0.0 - 21.0) *	11.5 (0.0 - 22.0) *

[a] n = 36, [b] n = 35

* Denotes a statistical difference in change over time for those patients who acquired AUST-02 ($P = \leq 0.05$)

Table 3.8 Clinical characteristics of patients with a recent AUST-06 acquisition compared to those who retained an AUST-06 infection for the duration of the study.

	Retention of AUST-06 strain n = 14		Acquisition of AUST-06 strain n = 17	
	Baseline	Final	Baseline	Final
Average age at collection, years, mean (95% CI)	26.1 (22.5 - 29.7)	31.2 (27.6 - 34.9)	30.3 (26.2 - 34.4)	35.4 (31.3 - 39.5)
Sex, males, n (%)	7 (50.0)		12 (70.6)	
CFTR, n (%)				
Minimal function	10 (71.4)		17 (100.0)	
Residual function	3 (21.4)		0 (0.0)	
Not classified	0 (0.0)		0 (0.0)	
Not available	1 (7.1)		0 (0.0)	
Lung Transplantation, n (%)	0 (0.0)		0 (0.0)	
Deceased, n (%)	0 (0.0)		0 (0.0)	
Pancreatic Insufficient, n (%)	13 (92.9)	13 (92.9)	16 (94.1)	16 (94.1)
Insulin Dependent Diabetes, n (%)	3 (21.4)	4 (28.6)	3 (17.7)	5 (29.4)
FEV_1 % predicted, mean (95% CI)	56.7 (46.7 - 66.7)	43.2 (32.7 - 53.7)	65.5 (53.7 - 77.3)	49.9 (41.8 - 57.9) [a]
Mean (95% CI) Annual FEV_1% predicted decline	-2.6 (-3.8 to -1.4)		-2.8 (-4.8 to -0.8) [a]	
BMI, mean (95% CI)	20.9 (19.2 - 22.6)	21.0 (19.0 - 22.9) *	22.3 (20.5 - 24.2)	22.7 (20.4 - 25.0) *
Mean (95% CI) Annual BMI decline	0.01 (-0.2 to 0.2)		0.07 (-0.2 to 0.3)	
Hospital Admissions - Respiratory, median (IQR)	2.0 (0.0 - 4.0)	3.0 (2.0 - 3.0)	1.0 (0.0 - 2.0)	3.0 (2.0 - 4.0)
Hospital Days - Respiratory, median (IQR)	20.0 (0.0 - 51.0)	39.0 (17.0 - 53.0)	10.0 (0.0 - 30.0)	42.0 (20.0 - 65.0)

[a] Results unavailable for one patient

* Denotes a statistical difference in change over time between patients who retained and acquired AUST-06 ($P = \leq 0.05$)

3.4.6 Patient outcomes

Eleven percent of the study population either died (n = 8) or required lung transplantation (n = 21) by the Final sample collection time point. Patients with an AUST-02 infection comprised the largest proportion of these patients (20%), while only one patient with an AUST-06 infection underwent lung transplantation. The rates of transplantation and death in the remainder of the population (AUST-01 [8.7%], minor shared [9.1%], unique [11.1%]) were very similar (Table 3.9).

Table 3.9 Rates of death and/or lung transplantation of patients at Final sample collection time point, stratified according to *Pseudomonas aeruginosa* genotype.

Strain	Survival n (%)	Death and/or lung transplantation n (%)
AUST-01 (n = 23)	21 (91.3)	2 (8.7)
AUST-02 (n = 50)	40 (80.0)	10 (20.0)
AUST-06 (n = 30)	29 (96.7)	1 (3.3)
Minor (n = 66)	60 (90.9)	6 (9.1)
Unique (n = 45)	40 (88.9)	5 (11.1)

The minor strain cohort, as presented in this table, is used as the reference group for the univariable analysis in Table 3.10.

The association between the composite endpoint (death or transplantation) and the presence of specific shared strain infection were assessed. This analysis showed that AUST-02 infection was associated with a higher risk of death and transplantation when compared to patients with minor shared strain infection. Following adjustment for the variables age (Odds Ratio (OR): 3.45, $P = 0.05$), FEV_1 % predicted (OR: 4.68, $P = 0.04$) and CFTR class (OR: 3.93, $P = 0.04$), AUST-02 remained significantly associated with risk of death and transplantation (Table 3.10A). Furthermore, this association held, even after adjusting for age and CFTR class (OR: 4.89, $P = 0.04$) and CFTR class and FEV_1% predicted (OR: 7.50, $P = 0.02$) (Table 3.10B). This association was not observed among patients infected with AUST-01 or unique strains.

Table 3.10 Univariate (A) and multivariate analysis (B) of Baseline patient data (n = 171) assessing the risks of dying or requiring lung transplantation, using *Pseudomonas aeruginosa* genotype and clinical variables.

A. Univariate Analysis

Strain type	N	n (%)	Strain		Age		FEV_1%pred		BMI		CFTR Class	
			OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Minor Only	46	5 (10.9)	Reference strain		Reference strain		Reference strain		Reference strain		Reference strain	
Unique Only	28	5 (17.9)	1.78 (0.47 - 6.81)	0.40	1.48 (0.37 - 6.00)	0.58	1.57 (0.32 - 7.67)	0.58	1.76 (0.44 - 7.01)	0.42	2.28 (0.53 - 9.85)	0.27
AUST-01 Only	17	2 (11.8)	1.09 (0.19 - 6.25)	0.92	1.27 (0.21 - 7.53)	0.79	1.21 (0.17 - 8.66)	0.85	1.19 (0.20 - 7.12)	0.85	1.53 (0.24 - 9.57)	0.65
AUST-02 Only	44	10 (22.7)	2.41 (0.75 - 7.74)	0.14	3.45 (1.00 - 11.90)	0.05	4.68 (1.04 - 20.90)	0.04	2.10 (0.63 - 6.94)	0.23	3.93 (1.08 - 14.24)	0.04

B. Multivariate Analysis

Strain type	N	n (%)	Age & CFTR Class		FEV_1%pred & CFTR Class	
			OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Minor Only	46	5 (10.9)	Reference strain		Reference strain	
Unique Only	28	5 (17.9)	1.48 (0.29 - 7.57)	0.64	1.88 (0.33 - 10.78)	0.48
AUST-01 Only	17	2 (11.8)	1.27 (0.18 - 9.17)	0.81	1.44 (0.18 - 11.74)	0.73
AUST-02 Only	44	10 (22.7)	4.89 (1.06 - 22.56)	0.04	7.50 (1.44 - 39.03)	0.02

n = The number of patients to die or undergo lung transplantation

N = The total number of patients with a particular *P. aeruginosa* genotype at Baseline sample collection time point

At Baseline there were no patients harbouring only the AUST-06 strain to die or required lung transplantation during the study period.

3.5 Discussion

This longitudinal study confirms that shared *P. aeruginosa* strains remain prevalent within TPCH ACFC patient population. Key findings of this investigation included that the frequency of AUST-02 infection declined, while in parallel, there was a significant and unexpected increase in the prevalence of AUST-06 infections. At Baseline sample collection the prevalence of AUST-01 and AUST-06 infections within this patient population were similar; however unlike AUST-06, AUST-01 infection rates remained stable for the study duration. Likewise, the prevalence of unique and minor shared strain infection remained unchanged during this period. Notably, the vast majority of patients that acquired AUST-06 were treated at TPCH ACFC throughout the study period. Taken together, these data suggest that there has been a surge in the transmission of the AUST-06 shared strain, but this appears to have occurred independently of other breakdowns in infection control within this CF centre.

Several studies examining the clinical impact of shared strain infection in CF have shown adverse outcomes, including increased lung function decline and nutritional status , increased risk of death or transplantation and increased hospitalisation and treatment requirements (Aaron *et al.* 2010; Al-Aloul *et al.* 2004; Jones *et al.* 2010; O'Carroll *et al.* 2004; van Mansfeld *et al.* 2016). Within the Australian CF population, two predominant shared strains, AUST-01 and AUST-02, have been identified and associated with an increased rate of death in young children (AUST-01) and increased treatment requirements in children and adults with CF (AUST-01 and AUST-02) (Armstrong *et al.* 2002; Griffiths *et al.* 2005; Kidd *et al.* 2013; O'Carroll *et al.* 2004). A national study of patients attending 18 Australian CF centres, demonstrated a prevalence of 22% and 18% for AUST-01 and AUST-02 strains, respectively. AUST-01 was isolated from patient's at all 18 centres and AUST-02 isolated from patients attending16 of the 18 centres. A third and less common shared strain, AUST-06, was initially identified in 2001 in patients from North Queensland and subsequent studies have confirmed that this strain is largely restricted to patients receiving care (current or previously) in Queensland (Kidd *et al.* 2013; O'Carroll *et al.* 2004).

As shown in other studies, these results also indicate that the clinical impact of shared strain *P. aeruginosa* infection is may be strain specific. The results presented here confirm that at time of clinical data collection, patients with shared stain infection were younger than those with a non-shared strain infection (Aaron *et al.* 2010; van Mansfeld *et al.* 2009). A Canadian study, examining the impact of a highly prevalent shared strain, Liverpool epidemic strain (LES), compared the clinical outcomes of patients harbouring LES to those harbouring a unique strain over a three year period. This study observed no difference in lung function at baseline, the rate of lung function decline,

antibiotic requirements or nutritional status. However, patients with LES were at greater risk of death or transplantation (Aaron *et al.* 2010). Another study undertaken in The Netherlands, reported that the prevalent shared strain within this CF population was associated with a greater usage of inhaled antibiotics yet, there was no difference when lung function decline or risk of death or transplantation were compared to patients free from shared strain infections (van Mansfeld *et al.* 2016). Here we confirm that one shared strain, AUST-02 is associated with increased death and transplantation, while a second shared strain, AUST-06 is associated with reduced lung function and increased hospitalisation.

This study also compared the clinical characteristics of patients who retained and acquired either AUST-02 or AUST-06 strains over time. Although under-powered, these data show that patients who retained a shared strain over time had a higher lung function at Baseline (AUST-02 only) and reduced decline in FEV_1 over time (AUST-02 and AUST-06). Furthermore, these patients had fewer hospital episodes over time, when compare to the new incidence cases. Similarly, these data suggest a trend to young patients with better lung function being more likely to acquire these shared strains, and following acquisition have greater clinical decline. Interestingly, data presented here suggested that the clinical outcomes of patients who retain a shared strain infection for an extended duration, appears to stabilise.

A number of clinically relevant observations were made when assessing this entire cohort. The annual lung function decline of all patients over the study duration was less than previously reported for similarly aged patients with *P. aeruginosa* infections (Konstan *et al.* 2012; Ren *et al.* 2012). Interestingly, clinical characteristics assessed at Baseline and Final sample collection time points remained stable over time. Additionally, patients from the TPCH with a stable shared strain infection were more likely to be younger, male, require lung transplantation, have a greater requirement for hospitalisation and have slightly greater lung function decline compared to those with a stable non-shared strain infection.

Focusing of on strain-specific outcomes, these analyses demonstrate that patients with shared strain infection were younger than those with a non-shared strain infection, which may account for the observation of a milder clinical phenotype and fewer hospitalisation episodes. Together these factors may limit exposure to source of infection due to lower requirements for hospital based care. The rates of co-morbidities were similar across the study for each of strain groups, however patients with AUST-06 has the highest rates of insulin-requiring CF related diabetes. The lung function of patients with AUST-01, AUST-02 and non-shared strains were not different at Baseline and study completion

with a similar decline in FEV_1 % predicted. In parallel, the rates hospitalisation was similar for patients within these strain groups.

During the national study, AUST-02 was the dominant shared strain at TPCH and this study confirms the previous results, despite the recent decline in AUST-02 prevalence (Kidd *et al.* 2013). Three factors may be responsible; firstly, the low rates of incidence cases since 2007, secondly, high rates of death or transplantation observed; and thirdly, an increase in the clinic population over this time, including patients without Pseudomonas infection (Ramsay *et al.* 2017). The prevalence of AUST-06 increased significantly during this study, largely due to new acquisitions occurring within TPCH ACFC. As strict cohort segregation was not logistically feasible during the observation period, patient-to-patient transmission of this strain is most likely route of acquisition. Supporting this hypothesis, AUST-06 patients appeared to be the most unwell patients (trend towards the poorest FEV_1 % predicted) with the highest hospitalisation rates of all the strain groups. The resulting high rates of patient attendance to clinic will have most likely increased exposure and acquisition of AUST-06 strains (Kidd *et al.* 2015; Knibbs *et al.* 2014; Wainwright *et al.* 2009). Additionally, environmental studies in Queensland have not isolated AUST-06 in other clinical or natural settings beyond patients with CF (Kidd *et al.* 2012). Despite these findings, the rates of death and transplantation for patients with AUST-06 were low (at least at this point in time). By way of contrast, patients with AUST-02 infection had the highest rates of death or transplantation during the study. Further work examining virulence determinants of AUST-06 strains should be undertaken to determine if there are intrinsic mechanisms which enhance acquisition and transmission. Subsequent strain-specific factors may be important to explain why AUST-06 increased in prevalence, whereas despite similar baseline prevalence AUST-01 remained stable throughout the study.

This study has a number of limitations and include; firstly, three *P. aeruginosa* isolates per sputum sample were collected annually for each patient which may have underestimated the diversity of strains within the airway of the participants. Previous work conducted by Kidd and colleagues demonstrated high level of genotype concordance when multiple isolates from a single patient undergo typing, suggesting that isolate selection is unlikely to impact the rates of strain diversity in this population (Kidd *et al.* 2013). Secondly, Pseudomonas genotyping was based on a 20-SNP assay which is able to accurately confirm the known shared strains infections within this patient population; however, the assay has lower discrimination than a MLST-based approach. Therefore, this technique may restrict the detection of recently emergent strains. Thirdly, the focus of this study was on the collection of clinical parameters, and was not designed to review patient-to-patient interactions which may have occurred during outpatient visits, inpatient hospitalisations or outside of the hospital

environment. This limits our ability to provide evidence regarding potential modes of acquisition such as person-to-person transmission. Fourthly, results analysed from this were generated from sputum samples collected annually and thus, the exact time of acquisition can only be estimated. As such, increased sample frequency and invasive sampling approaches may have provided a more accurate means of determining the point at which each shared strain was acquired. Finally, as only the 34 patients who acquired a shared strain infection had comprehensive analyses of *P. aeruginosa* isolates collected annually between Baseline and Final collection time points to confirm date of acquisition, some incidence cases may be underestimated as specific infections could be intermittent.

This current study reports an increase in the prevalence of the AUST-06 strain and a decline in AUST-02 strain. Within this patient cohort AUST-02 strain infection was associated with an increased risk of death or transplantation, whereas, only one patient co-infected with the AUST-06 strain (and AUST-02) required lung transplantation. However, a trend towards lower lung function and increased hospital requirements was noted in patients with an AUST-06 infection. These results demonstrate the complexity of infection and how dynamics such as patient population and clinical outcomes can influence prevalence. Nevertheless, the lack of an environmental reservoir and the increase of shared strains implicate patient to patient transmission as the driving force behind these infections. Evidence of strain diversity demonstrated by the high rates of non-shared strains confirms that environmental acquisition is also occurring within a portion of patients. Since the completion of this study a purpose-built CF inpatient facility has been commissioned allowing enhanced patient segregation with single room accommodation for all patients. Surveillance of the impact of the new facility and enhanced infection control procedures is underway.

Chapter 4: Phenotypic characteristics of *Pseudomonas aeruginosa*

4.1 Abstract

Background: The environmental bacterium *P. aeruginosa* is the most prevalent pathogen isolated from respiratory secretions in people with cystic fibrosis (CF) and infection is associated with adverse clinical outcomes. Studies have demonstrated that during establishment of chronic airway infection in people with CF, *P. aeruginosa* undergoes a number of phenotypic changes including biofilm development, loss of motility and the expression of mucoidy colonial phenotype. However, few studies have phenotypically characterised isolates obtained from a range of environmental niches and clinical conditions under varied atmospheric conditions with the aim of determining pathoadaptive traits unique to isolates derived from the CF airways.

Methods: A total of 167 *P. aeruginosa* isolates collected from the environment, animal, non-CF human infections and people with CF underwent a number of phenotypic tests to determine colonial morphology, motility and initial biofilm adhesion under different atmospheric conditions (aerobic, microaerophilic and anaerobic conditions).

Results: Overall, most isolates displayed pigmented, non-mucoid morphotypes. Phenotypically, isolates obtained from the environment, animals and non-CF patients were similar, all displaying high levels of motility and adhesion capabilities. In contrast, CF isolates were predominantly non-motile and non-adherent under each different atmospheric condition. The greatest difference in phenotypes was noted when comparing CF and environmental isolates. Overall, motility and adhesion was negative associated with growth under anaerobic conditions compared with aerobic conditions.

Conclusions: This work suggests the CF isolates are intrinsically different from isolates collected from all other niches. Despite an overall reduction in the phenotypic characteristics displayed by the CF isolates, non-shared strains displayed greater phenotypic diversity compared to shared strains. This study demonstrates that a phenotypic gradient from poorly niche adapted (environmental isolates) to highly niche adapted (shared CF strains) can be demonstrated when assessing isolates from varied settings.

4.2 Introduction

Pseudomonas aeruginosa is an environmental bacterium possessing a range of pathoadaptive traits and virulence mechanisms which promote colonisation, survival, and proliferation (Cullen and McClean 2015; Hauser *et al.* 2011; Hogardt and Heesemann 2010; Sousa and Pereira 2014; Winstanley *et al.* 2016). For people with cystic fibrosis (CF), *P. aeruginosa* is a highly prevalent pathogen isolated from respiratory secretions and is associated with increased mortality and exacerbations, accelerated rate of lung function decline and decreased quality of life (Elborn 2016; Ratjen *et al.* 2015). Within the lungs, *P. aeruginosa* utilises these adaptive traits to evade the host immune system in order to establish a chronic infection. These mechanisms include, but are not limited to, loss of motility and type III secretion, enhancement of a mucoid phenotype, increased antibiotic resistance, development of small colony variants, changes to lipopolysaccharide, O-antigen and pyocyanin expression, growth within sessile community formations and defects to bacterial communication and hypermutability (Cullen and McClean 2015; Folkesson *et al.* 2012; Winstanley *et al.* 2016). Many of these pathoadaptive traits are essential to support the formation of bacterial communities, known as biofilms. Growth within biofilms is preferential as structural and metabolic features provide protection from the host immune system and antimicrobial agents and peptides (Alhede *et al.* 2014).

Typically, colonisation of the CF airways occurs in childhood and is characterised by intermittent infection which can be initially eradicated by the use of anti-pseudomonal antibiotics (Folkesson *et al.* 2012). Genotypically, isolates from early infections are heterogeneous and patients will usually harbour individual strains (Kidd *et al.* 2015; Ranganathan *et al.* 2013). Over time *P. aeruginosa* will often re-infect and ultimately establish chronic infection within the airway (Folkesson *et al.* 2012). Over the past 20 years, strain typing of isolates has shown that genotypically indistinguishable (shared) strains can be isolated from unrelated patients and in some instances are associated with adverse health outcomes (Aaron *et al.* 2010; Armstrong *et al.* 2002; Cheng *et al.* 1996; Kidd *et al.* 2013; O'Carroll *et al.* 2004; Scott and Pitt 2004; van Mansfeld *et al.* 2009). Environmental reservoirs for shared *P. aeruginosa* strains have not been identified, suggesting niche adaptation within the CF airway and the potential for acquisition by cross-infection with highly adapted bacteria (Jones *et al.* 2003; Kidd *et al.* 2012).

Phenotypic analysis of *P. aeruginosa* has primarily focused on describing characteristics of isolates obtained from people with CF. Overall, these analyses indicate that isolates obtained from early and intermittent infections possess a range of virulence factors, show a non-mucoid and pigmented appearance, and have propensity for aerobic and free-living (or planktonic) growth. Whereas, isolates

obtained from chronically infected patients, including those with a shared strain will often be phenotypically distinct from non-shared strains or those obtained during intermittent infection. (Costerton *et al.* 1999; Head and Yu 2004; Hogardt and Heesemann 2010; Hunter *et al.* 2012; Li *et al.* 2005; Mahenthiralingam *et al.* 1994; Manos *et al.* 2013; Mayer-Hamblett *et al.* 2014; Mayer-Hamblett *et al.* 2014; Murray *et al.* 2010). Typically, these isolates will be non-motile, display a mucoid appearance, grow within biofilm formations, and exhibit increase resistance to many anti-pseudomonal antibiotics (Lee *et al.* 2005; Mahenthiralingam *et al.* 1994; Manos *et al.* 2013; Mayer-Hamblett *et al.* 2014; Tingpej *et al.* 2007). However, not all isolates from people with CF will conform to these phenotypic generalisations. Isolates from initial and early infection have been shown to display mucoid, and motile phenotypes (Burns *et al.* 2001; Mayer-Hamblett *et al.* 2014), and conversely shared strains and *P. aeruginosa* isolated from chronically infected patients can display non-mucoid phenotypes (Jeukens *et al.* 2014; Manos *et al.* 2013).

Compared to isolates from people with CF, those obtained from non-CF human infections and the natural environment often demonstrate enhance phenotypic characteristic, although an extensive study of these isolates has not been conducted (Head and Yu 2004; Mahenthiralingam *et al.* 1994; Murray *et al.* 2010). In contrast to non-CF isolates, *P. aeruginosa* isolated from people with CF have been frequently exposed to antibiotics and host immune factors resulting in highly niche adapted strains in chronic lung infection. Therefore, recent studies sought to characterise the evolution strategy of *P. aeruginosa* including pathways, which enable survival and persistence within the CF airways (Jeukens *et al.* 2014; Manos *et al.* 2009; Manos *et al.* 2008; Silby *et al.* 2011; Stover *et al.* 2000). Whether similar mechanisms are exhibited by Australian *P. aeruginosa* CF strains is currently unknown.

Therefore, to address this knowledge gap, through the use of phenotypic assays, this study aimed to characterise and compare adaptive traits of a large number of isolates collected from in different clinical and environmental niches with the aim of identifying particular mechanisms which may be advantageous during niche adaptation. Interestingly, from these investigations I observed that isolates from people with CF possessed a very distinct phenotype to those of isolates from varied clinical and environmental settings. This work highlights that despite lacking mechanisms previously associated with the establishment of infection, the shared CF strain, AUST-02, is still commonly isolated from chronically infected patients.

4.3 Materials and Methods

4.3.1 Isolate Selection

One hundred and sixty-seven *P. aeruginosa* isolates obtained from environmental settings and clinical sources found in South East Queensland, Australia, as previously described by Kidd and colleagues, were selected for testing (Kidd *et al.* 2013; Kidd *et al.* 2012). They included isolates sampled from the environment (n = 34), animals (n = 14), non-CF human infections (n = 20), and sputum samples from people with CF (n = 99). *P. aeruginosa* strain PAO1 (ATCC 15692), originally isolated from cutaneous infection in a patient who had suffered burns and supplied by Professor John Mattick from the University of Queensland (UQ) was used as a reference strain (Holloway 1955).

Thirty-three environmental isolates were collected in 2009 from urban, rural and industrial regions of three local riverine systems and one additional isolate was obtained from a household sink drain (Kidd *et al.* 2012). Animal isolates included five isolates which were obtained from UQ Veterinary School and nine isolates from a private animal pathology service. All isolates were collected during 2007 and 2008 and included samples collected from ears (n = 8), respiratory secretions (n = 2), urine (n = 1), milk (n = 1) and the reproductive tract (n = 2). Animal species included: canine (n = 9), equine (n = 2), feline (n = 2), and caprine (n = 1). The non-CF clinical specimens included isolates collected from respiratory secretions (n = 7), including three from patients with non-CF bronchiectasis), blood (n = 5), wound (n = 2), urine (n = 4) and ear (n = 2) infection samples. The three non-CF bronchiectasis respiratory samples were collected from patients who were cared for at The Prince Charles Hospital (TPCH) and the remaining 17 samples were collected from patients who attended the Royal Brisbane and Women's Hospital (RBWH) (2002 - 2008).

Ninety-six of the 99 isolates obtained from people with CF were collected between 2002 and 2014 from adults (75%) and children (25%) whilst participating in a national multi-centre cross sectional prevalence study (Kidd *et al.* 2013). All patients resided in South Eastern Queensland and attended CF clinics based at one of four CF centres. Three isolates; Canadian *P. aeruginosa* strain A 01-127-A (ST-146) isolated from a person with CF and supplied by Dr Shawn Aaron from University of Ottawa, Ontario; and *P. aeruginosa* strains CF1 (ST-146) and CF7 (ST-146) both clinical isolates obtained from people with CF and supplied by Ms Deirdre Gilpin and Professor Stuart Elborn from Queen's University, Belfast were included in the analysis.

Collectively, 57 individual STs were represented within this isolate collection. This included 20 isolates from five STs found across all four different ecological niches (i.e. ST-155, ST-179, ST-253, ST-266, ST-381) and 56 isolates representing the two major CF shared strains found in

people with CF residing in Queensland, Australia. (ST-775 [AUST-02, n = 50]; ST-801 [AUST-06, n = 6]) (Kidd *et al.* 2012) (Figure 4.1).The remaining 91 isolates was comprised of singletons, with unique ST, and pairs and triplicates sharing individual STs.

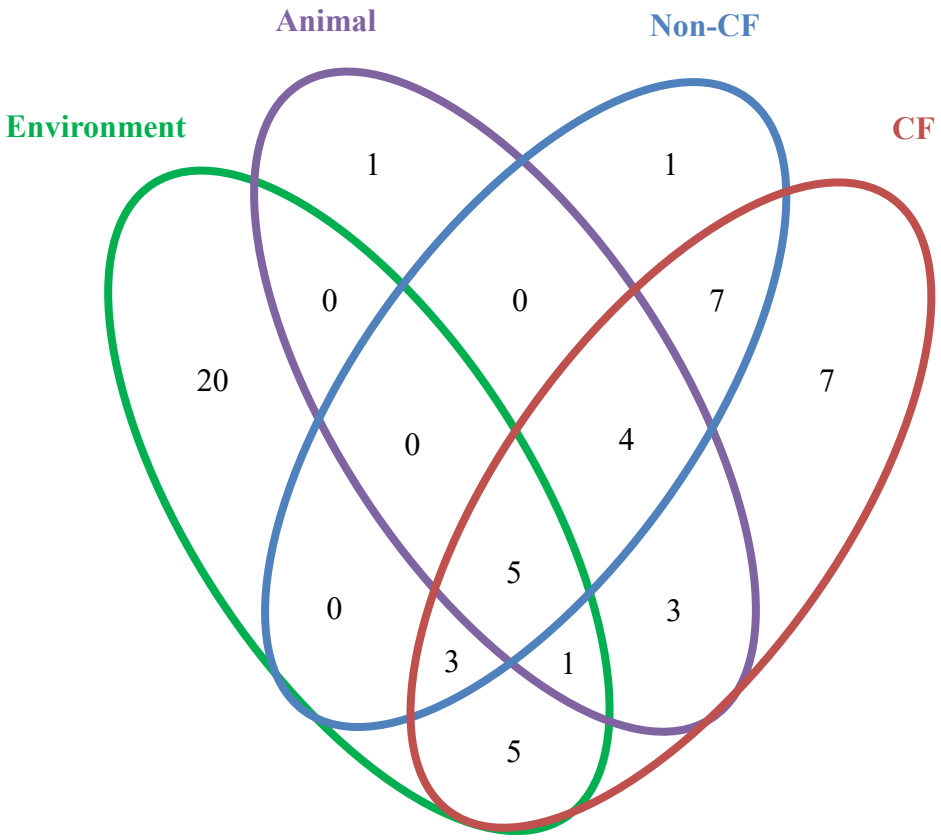


Figure 4.1 Distribution of individual sequence types (ST) selected for analysis in the motility and adhesion assays.

4.3.2 Sample collection

Clinical samples were cultured by Queensland Pathology diagnostic microbiology laboratories (human non-CF and CF specimens) and UQ Veterinary School (animal specimens). Standardised phenotypic and biochemical techniques, as described previously, were used for the identification of *P. aeruginosa* (Kidd *et al.* 2009). When *P. aeruginosa* was identified, isolates selected for inclusion in this study and forwarded onto the research laboratory for storage at -80 °C until required for further testing (Kidd *et al.* 2013). As described by Kidd and colleagues, environmental samples of surface water and air-water interface swabs were collected from local riverine systems and identified. Each water sample was filtered (47 mm, 0.45 µm cellulose filter) prior to microbiological analysis. All samples were cultured onto M-PA-C agar (BD BBL™, North Ryde, Australia) and incubated aerobically at 42 °C for up to 48-hours (Kidd *et al.* 2012).

4.3.3 Isolate storage and retrieval

Each isolate was sub-cultured onto nutrient agar (Oxoid Australia Pty. Ltd., Adelaide, Australia) to assess purity. Isolates were then prepared for storage as follows. Each bacterium was subcultured into Luria-Bertani, Miller broth (Oxoid Australia Pty. Ltd., Adelaide, Australia) (LB broth) and incubated overnight at 37 °C. From this inoculum a 15% glycerol (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia) stock was prepared and stored at -80 °C until required (Kidd *et al.* 2009). When isolates were required for further testing, they were retrieved from storage and cultured onto Luria-Bertani, Miller agar (BD Difco™, North Ryde, Australia) (LB agar) and incubated at 37 °C for 24-hours. A subsequent sub-culture, using the same conditions, was performed prior to analysis (Syrmis *et al.* 2013).

4.3.4 DNA Extraction

Heat denatured bacterial DNA was prepared as follows. A 1.0 McFarland suspension was prepared from a 24 hour single-colony subculture in 2.0 mL of water (Baxter Healthcare Pty. Ltd., Coorparoo, Australia) and heated for 20 minutes at 95 °C. Following a short period of cooling, the suspensions were centrifuged at 14,000 rpm for 4 minutes. The supernatant was then stored at -80 °C prior to genotyping (Anuj *et al.* 2009; Syrmis *et al.* 2013).

4.3.5 Confirmatory Identification

All isolates underwent the *P. aeruginosa* specific real time duplex PCR assay, targeting the *ecfX* and *gyrB* genes, to confirm identification (Anuj *et al.* 2009). Each PCR reaction mix was comprised of, 12.5 µL of Qiagen Quantitect probe master mix, 10 pmol/µL each forward and reverse primer (Table 4.1), 20 pmol/µL of each TaqMan probe (Table 4.2) and 2 µL of DNA template, made up to a final reaction volume of 25 µL using water.

The thermal cycling conditions were as follows. An initial hold of one cycle at 95 °C for 15 minutes, followed by 50 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds and one final cooling cycle of 40 °C for 30 seconds. Cycling and amplicon detection was performed on a LightCycler®480 instrument. The fluorescent probes for *ecfX* and *gyrB* were labelled with Yakima Yellow (YAK) and a fluorescein, FAM, so they could be detected in the JOE (523 - 568 nm) and FAM (483 - 533 nm) channels, respectively (Anuj *et al.* 2009; Kidd *et al.* 2009). Control isolates, including *Achromobacter xylosoxidans* LMG 1863 and a representative of control strains, AUST-01 and AUST-02, were included in each assay.

Table 4.1 Primers used in *P. aeruginosa* specific real time duplex PCR assay.

Name	Primer Sequence 5'-3'
ecfX-fwd	CGCATGCCTATCAGGCGTT
ecfX-rvs	GAACTGCCCAGGTGCTTGC
gyrB-fwd	CCTGACCATCCGTCGCCACAAC
gyrB-rvs	CGCAGCAGGATGCCGACGCC

Table 4.2 Probes used in *P. aeruginosa* specific real time duplex PCR assay.

Name	Probe Sequence 5'-3'
ecfX-probe	JOE-ATGGCGATTTGCTGCGCTTCCT-BHQ1
gyrB-probe	FAM-CCGTGGTGGTAGACCTGTTCCCAGACC-BHQ1

4.3.6 Multilocus sequence analysis

All reagents, primers and thermal cycling conditions used were identical to those found on the *Pseudomonas aeruginosa* MLST website (Jolley and Maiden 2010; *Pseudomonas aeruginosa* PubMLST Database 2012). MLST sequencing was performed by the Australian Genome Research Facility (AGRF). DNA sequences were analysed using Sequence Scanner v1.0 and Vector NTI Advance 11.0 software as described previously by Kidd and colleagues (Kidd *et al.* 2011).

4.3.7 Morphology

Visual inspection of the bacterial colonies following overnight growth on LB agar was performed to determine mucoid and pigment production (Mayer-Hamblett *et al.* 2014; Tingpej *et al.* 2007). Pigment production included the presence of pyocyanin (blue-green) and pyomelanin (brown) (Appendix 3.2).

4.3.7.1 Motility Assays

Motility assays were performed on all isolates listed in Appendix 3.1. Three technical replicates (repeated measures of the same bacterial suspension) were tested to determine swimming, swarming and twitching capabilities. All motility assays were performed under aerobic (21% O_2, 0.04% CO_2), anaerobic (0% O_2, 20% CO_2) and microaerophilic (5% O_2, 15% CO_2) atmospheric conditions and incubated for 24-hours at 30 °C.

4.3.7.2 Swimming Motility

Isolates were grown on LB agar at 37 °C for 24-hours prior to preparation of a single colony subculture into LB broth. Swim media was prepared and inoculated as follows, LB broth supplemented with 0.3% Bacto agar (w/v) (BD Bacto™, North Ryde, Australia) was inoculated using a 5 µL volume of the overnight LB culture into the centre of the agar to demonstrate swimming motility (O'May and Tufenkji 2011; O'May *et al.* 2006; Rashid and Kornberg 2000) (Appendix 3.3A).

4.3.7.3 Swarming Motility

Isolates were grown on LB agar at 37 °C for 24-hours prior to preparation of a single colony subculture into LB broth. Swarm media was prepared and inoculated as follows, nutrient broth (Oxoid Australia Pty. Ltd., Adelaide, Australia) supplemented with 0.5% Bacto agar (w/v) and 0.5% Glucose (w/v) (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia) was surface inoculated with a 5 µL volume of the overnight LB culture to demonstrate swarming motility (O'May and Tufenkji 2011; O'May *et al.* 2006; Rashid and Kornberg 2000) (Appendix 3.3B).

4.3.7.4 Twitching Motility

All isolates were initially grown on LB agar at 37 °C for 24-hours. LB broth supplemented with 1% Bacto agar (w/v) was prepared to a depth of 3 mm to assess twitching motility (O'May *et al.* 2006; Rashid and Kornberg 2000). A single colony isolated from the aforementioned LB agar subculture was stab inoculated to the plastic/agar interface at the base of the petri dish containing the twitch agar using a sterile toothpick. Following 24-hours incubation, the zone of growth was measured (standard methodology). After additional 24-hours incubation the media was dehydrated and removed. The zone of growth, adhered to the petri dish, was then stained with 0.25% Coomassie® Brilliant Blue (w/v) (Bio-Rad Laboratories Pty. Ltd., Gladesville, Australia) for 15 minutes at room temperature to enable measuring (extended methodology) (Alm and Mattick 1995; O'May and Tufenkji 2011). Comparisons of the zone sizes collected from the standard and extended methodologies were conducted to determine if measurement through the agar alone was sufficient to accurately determine a positive result (Appendix 3.3C).

4.3.7.4.1 Interpretation of motility assays

The zone of growth (diameter mm) was measured for all isolates after 24-hours incubation. From these individual measurements (in triplicate for each bacteria tested), the mean area ($A = \pi r^2$) to represent each isolate, was calculated to determine the motility characteristics. The following criteria were used to determine motility. Firstly, the area of growth had to be ≥ 10% of the control PAO1 isolate (Murray *et al.* 2010), and/or the total area of growth must be ≥ 20 mm^2 to be considered motile. Isolate growth determined to be ≤ 20 mm^2 is equal to the size of the initial inoculum, therefore it was

concluded that whilst the bacteria was able to grow they were non-motile (Lee *et al.* 2005; Mahenthiralingam *et al.* 1994).

4.3.7.5 Microtitre plate-based assay

Bacterial adhesion of all isolates (Appendix 3.1) was assessed using a microtitre plate-based assay (O'Toole 2011). Overnight bacterial cultures in LB broth were adjusted to 0.5 McFarland (1.5×10^8 colony forming units (CFU)/ml) and further diluted to a 1 in 100 suspension using LB broth. Three wells (technical replicates) of a round bottom polystyrene microtitre plate (Greuber Bio-One International GmbH, Kremsmünster, Austria) were inoculated with 200 µL of each bacterial suspension and incubated for 24-hours under aerobic, anaerobic and microaerophilic atmospheric conditions at 30 °C. Following incubation the microtitre plate was inverted and the bacterial suspension was shaken out. The plate was then submerged in water and washed three times, with the water being smacked out of the plates in between each wash. To stain the adhered growth, 200 µL of 0.1% crystal violet (Merck Millipore, Merck and Co., Bayswater, Australia) was dispensed into each well and incubated at room temperature for 30 minutes. Following incubation the stain was smacked out of the plate and washed three times in water. To quantify the amount of adhered bacterial growth 200 µL of 95% ethanol was dispensed into each well and incubated for 30 minutes at room temperature. A plate reader was used to quantify absorbance using optical density read at optical density 595 nm (OD_{595nm}), using a blank of uninoculated LB broth (O'Toole 2011) (Appendix 3.4).

4.3.7.5.1 Interpretation of microtitre plate assay

All isolates were cultured in triplicate (technical replicates) and a duplicate of each assay was then performed on a second 96-well plate to account for inter-run variability. Quantification of the bacterial adhesion was determined using the equations proposed by Stepanovic and colleagues (Table 4.3) where the OD_{595nm} of the test and the blanks are compared (Stepanovic *et al.* 2000; Stepanovic *et al.* 2007). For this analysis those that returned a weak, moderate or strong result were determined to be positive for adhesion.

Table 4.3 Equation for the quantification of bacterial adherence as determined by the microtitre assay.

Calculation	Result
OD ≤ Odc	Absent
Odc < OD ≤ 2 x Odc	Weak adhesion to the microtitre plate
2 x ODC < OD ≤ 4 x ODC	Moderate adhesion to the microtitre plate
4 x Odc < OD	Strong adhesion to the microtitre plate

Abbreviations: OD, Optical density read at OD_{595nm}; Odc, average OD of 95% ethanol blanks + (3 x SD); SD, standard deviation of the 95% ethanol blanks.

4.3.7.6 Anaerobic and microaerophilic atmospheric conditions

Media prepared for these assays were supplemented with 10% Potassium Nitrate (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia) to support growth under anaerobic and microaerophilic conditions (O'May *et al.* 2006; Yoon *et al.* 2002). Reduced atmospheric conditions (anaerobic and microaerophilic) were obtained using the GENbox commercial system as developed by bioMérieux (BioMerieux Australia Pty. Ltd, Murarrie, Australia). Either GENbox Anaer or GENbox Microaer generator sachets were included when plates were incubated in the airtight box. According to manufacturer's guidelines these sachets will produce the desired atmospheric condition within one hour of activation. Included in each batch was a colour indicator strip, as supplied by bioMérieux and *P. aeruginosa* inoculated onto a blood agar plate, without supplementation. The desired atmospheric conditions were confirmed by the colour change of the indicator strip and subdued *P. aeruginosa* growth.

4.3.8 Statistical Analyses

Categorical data (i.e. niche versus phenotype in different atmospheric conditions) were examined using a Chi-square with Yates continuity correction. Bonferroni corrections were performed to adjust for multiple comparisons. Simple regression and correlation was used to examine the association between motility (Pearson correlation coefficient) and adhesion assays (Spearman's rank correlation coefficient). Intraclass correlation coefficients (ICC) were calculated using two-way random effects with a consistency metric. For variables that showed considerable skewness, data were transformed the (log (1+x) and performed the linear modelling on the log scale). Comparisons were not adjusted for multiple testing. Generalised Bland-Altman plots followed the extension suggested by Jones and colleagues (Jones *et al.* 2011). These plots represent a graphical method for assessing agreement with the mean between multiple observers using continuous measures. Each dot in these plots represents a single isolate with up to three replicates accounted for. Statistical analyses were performed using the commercially available statistical packages; R and SPSS version 22 (IBM Corp. Released 2013.

IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp, http://www.R-project.org). A *P* value ≤ 0.05 indicated statistical significance.

4.4 Results

4.4.1 Morphotype

The CF isolates were less often pigmented compared with environmental isolates and were less often mucoid than the non-CF human isolates. The non-shared CF strains tended to be more pigmented and more often mucoid compared to AUST-02 and AUST-06 strains (Table 4.4).

Table 4.4 Colonial morphotypes of *Pseudomonas aeruginosa* isolates collected from human, animal and environmental sources, n (%).

Ecological niche or strain	Colonial morphotype	
	Mucoid	Pigmented
Panel A. All isolates[§]		
Environmental (n = 34)	8 (23.5)	27 (79.4) [a]
Animal (n = 14)	4 (28.6)	8 (57.1)
Non-CF (n = 20)	8 (40.0) [a]	10 (50.0)
Cystic fibrosis (n = 99)	13 (13.1) [a]	42 (42.4) [a]
Panel B. Cystic fibrosis isolates[#]		
Non-Shared Strains (n = 33)[*]	6 (18.2)	18 (54.5)
AUST-02 (n = 50)	3 (6.0)	21 (42.0)
AUST-06 (n = 6)	0 (0.0)	0 (0.0)
AUST-01 (n = 6)[†]	4 (66.7)	3 (50.0)
LES (n = 4)[†]	0 (0.0)	0 (0.0)

[§] Bonferroni correction adjusted for 6-way comparison.

[#] Bonferroni correction adjusted for 3-way comparison (Non-Shared, AUST-02 and AUST-06).

[*] Strains found only in individual patients or < 10 of patients in attendance at TPCH.

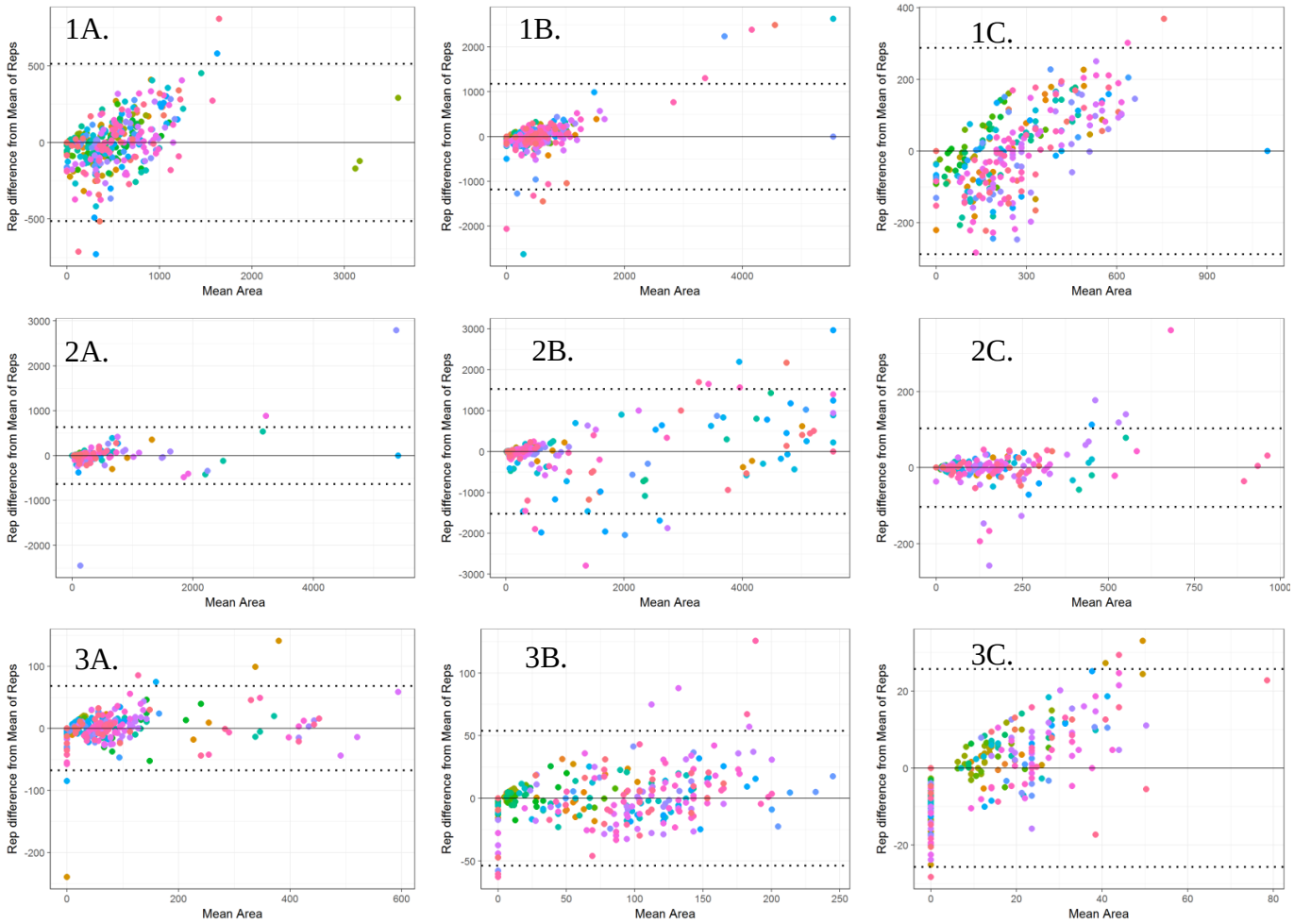
[†] Due to small numbers and as the focus of this study was on the prevalent shared strains identified within the local TPCH CF population, these strains were not included in analysis.

[a] Values within columns showing a letter in common were significantly different ($P \leq 0.05$) following Bonferroni correction.

4.4.2 Motility Assays

4.4.2.1 Reproducibility of the motility assays

Limits of agreement measures for each motility assessed in aerobic conditions demonstrated excellent reproducibility (swim motility, ICC: 0.937 [95% CI: 0.915 to 0.954], $P < 0.001$; swarm motility, ICC: 0.943 [95% CI: 0.926 to 0.957], $P < 0.001$; twitch motility, ICC: 0.973 [95% CI: 0.965 to 0.980], $P < 0.001$). Similar results were also obtained when assessed using microaerophilic and anaerobic conditions (Figure 4.2). In the majority of cases less than 10% of strain comparisons fell outside of ±1.96SD of the mean. High levels of agreement were also observed within groups when niche, atmospheric conditions and motility was more closely analysed (Appendix 3.5).



1A. ICC: 0.973 (95% CI: 0.915 to 0.954), $P < 0.001$
1B. ICC: 0.696 (95% CI: 0.598 to 0.773), $P < 0.001$
1C. ICC: 0.857 (95% CI: 0.813 to 0.891), $P < 0.001$

2A. ICC: 0.943 (95% CI: 0.926 to 0.957), $P < 0.001$
2B. ICC: 0.966 (95% CI: 0.956 to 0.974), $P < 0.001$
2C. ICC: 0.972 (95% CI: 0.963 to 0.978), $P < 0.001$

3A. ICC: 0.973 (95% CI: 0.965 to 0.980), $P < 0.001$
3B. ICC: 0.964 (95% CI: 0.953 to 0.972), $P < 0.001$
3C. ICC: 0.702 (95% CI: 0.615 to 0.773), $P < 0.001$

Figure 4.2 Reproducibility assessment of 1) swim, 2) swarm and 3) twitch motility under a) aerobic, b) microaerophilic and c) anaerobic conditions using a generalised Bland-Altman Plot.

Bounds for this plot are ISO-defined reproducibility limits, defined as ±1.96 SD. Each isolate, tested in triplicate, is represented by three dots of an individual colour.

Note: Different values for both the *x* and *y* axis as determined by the specific motility and atmospheric conditions being analysed.

4.4.2.2 CF isolates had reduced motility compared to isolates from other clinical and environmental sources

Overall, isolates cultured from the patients with CF demonstrated reduced swimming, swarming and twitching motilities under each atmospheric condition when compared with all other isolates tested. More specifically, the CF isolates displayed significantly reduced swimming, swarming and twitching capabilities across all atmospheric conditions compared with environmental isolates. Apart from anaerobic twitching motility ($P = 0.090$), all of the non-CF isolates produced significantly larger zones of motility when compared to those generated by CF isolates. Overall, animal and CF isolates were the most similar (to each other), with only swarm (aerobic and microaerophilic) and twitch (aerobic, microaerophilic, anaerobic) motilities achieving a statistical difference (Table 4.5, Panel A). Overall, these analyses demonstrate that niche adapted CF strains possess similar motility traits. These data are reflective of previous reported results which demonstrate that isolates obtained from chronic infections display reduced motility compared to those from intermittent infections.

4.4.2.3 Variable motility results of shared and non-shared strains obtained from people with CF

When compared to the non-shared CF strains, AUST-02 displayed a significant reduction in all motilities. In contrast, the non-shared CF and AUST-06 strains shared similar motility characteristics across all atmospheric conditions. AUST-06 displayed reduced motility characteristics compared to isolates obtained from the environment, animal and non-CF settings, however these results did not always reach a significant difference. Whereas, AUST-02 displayed significantly reduced motility when compared to isolates collected from these niches. Despite the phenotypic heterogeneity noted when comparing the shared strains with isolates from all other niches, the AUST-02 and AUST-06 strains displayed very similar motility characteristics to each other. (Table 4.5, Panel B).

4.4.2.4 Environmental, animal and non-CF isolates display similar motility capabilities

Environmental, animal and non-CF human isolates displayed enhanced motility characteristics, with the majority positive in all assays (Table 4.5, Panel A).

4.4.2.5 Isolates had impaired motility under anaerobic conditions

The majority of isolates demonstrated a reduction in swim, swarm and twitch motilities when tested under anaerobic conditions, compared to characteristics observed in aerobic conditions (Table 4.5). Twitch motility demonstrated the greatest reductions when grown in anaerobic conditions. Environmental ($P < 0.001$), non-CF ($P = 0.024$), all CF ($P = 0.001$) and non-shared CF ($P < 0.001$)

isolates all reported significant reductions. Furthermore, swimming motility of the AUST-02 strain also displayed a significant reduction when tested anaerobically ($P = 0.010$).

Interestingly, rather than being negatively correlated with motility, an increase in swarming was noted when the environmental isolates were tested in anaerobic conditions ($P = 0.036$). When tested in aerobic conditions 68% of the isolates displayed a positive swarming motility, whereas, this increased to 91% of isolates that swarmed under anaerobic conditions. (Appendix 3.6).

4.4.2.6 Twitch results

In line with the swim and swarm motility assays, the twitch motility assays were initially assessed after a 24-hour incubation period. Due to difficulties measuring the area which the bacteria travelled away from the inoculation site through the agar, the medium was dehydrated, removed and the zone of motility stained to more accurately assess twitch capabilities. The additions to the methodology resulted in larger zones for all isolates apart from AUST-06. This is most likely due to the small numbers of isolates tested. Despite this a significant change in the result was only reported for nine comparisons (16.7%) (Appendix 3.7).

Table 4.5 Motility assays tested on *Pseudomonas aeruginosa* isolates collected from human, animal and environmental sources, conducted under various atmospheric conditions, n (%).

Ecological niche or strain	Swim-Positive			Swarm-Positive			Twitch-Positive		
	O_2	MA	AnO_2	O_2	MA	AnO_2	O_2	MA	AnO_2
Panel A. All isolates[§]									
Environmental (n = 34)	34 (100.0) [a]	33 (97.1) [a]	32 (94.1) [a]	23 (67.6) [a]	19 (55.9) [a]	31 (91.2) [acd]	28 (82.4) [a]	33 (97.1) [ade]	10 (29.4) [a]
Animal (n = 14)	12 (85.7)	12 (85.7)	9 (64.3)	9 (64.3) [b]	9 (64.3) [b]	5 (35.7) [c]	10 (71.4) [b]	9 (64.3) [bd]	4 (28.6) [b]
Non-CF (n = 20)	19 (95.0) [b]	18 (90.0) [b]	18 (90.0) [b]	13 (65.0) [c]	13 (65.0) [c]	10 (50.0) [bd]	12 (60.0) [c]	12 (60.0) [ce]	4 (20.0)
Cystic fibrosis (n = 99)	49 (50.0) [^ab]	49 (50.0) [^ab]	37 (37.4) [ab]	22 (22.2) [abc]	9 (9.1) [abc]	16 (16.1) [ab]	18 (18.2) [abc]	20 (20.2) [abc]	3 (3.0) [ab]
Panel B. Cystic fibrosis isolates[#]									
Non-Shared Strains (n = 33)*	23 (69.7) [y]	22 (66.7)[y]	20 (60.6) [y]	15 (45.5) [y]	9 (27.3) [y]	13 (39.4) [y]	16 (48.5) [y]	18 (54.5) [y]	1 (3.0)
AUST-02 (n = 50)	18 (36.0) [y]	16 (32.0)[y]	6 (12.0) [yz]	5 (10.0) [y]	0 (0.0) [y]	2 (4.0) [y]	2 (4.0) [y]	1 (2.0) [y]	1 (2.0)
AUST-06 (n = 6)	4 (66.7)	5 (83.3)	6 (100.0) [z]	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	1 (16.7)
AUST-01 (n = 6)[†]	2 (33.3)	3 (50.0)	4 (66.7)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
LES (n = 4)[†]	2 (50.0)	3 (75.0)	1 (25.0)	2 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

[§] Bonferroni correction adjusted for 6-way comparison.

[#] Bonferroni correction adjusted for 3-way comparison (Non-Shared, AUST-02 and AUST-06).

[^] n = 98

[*] Strains found only in individual patients or < 10 of patients in attendance at TPCH.

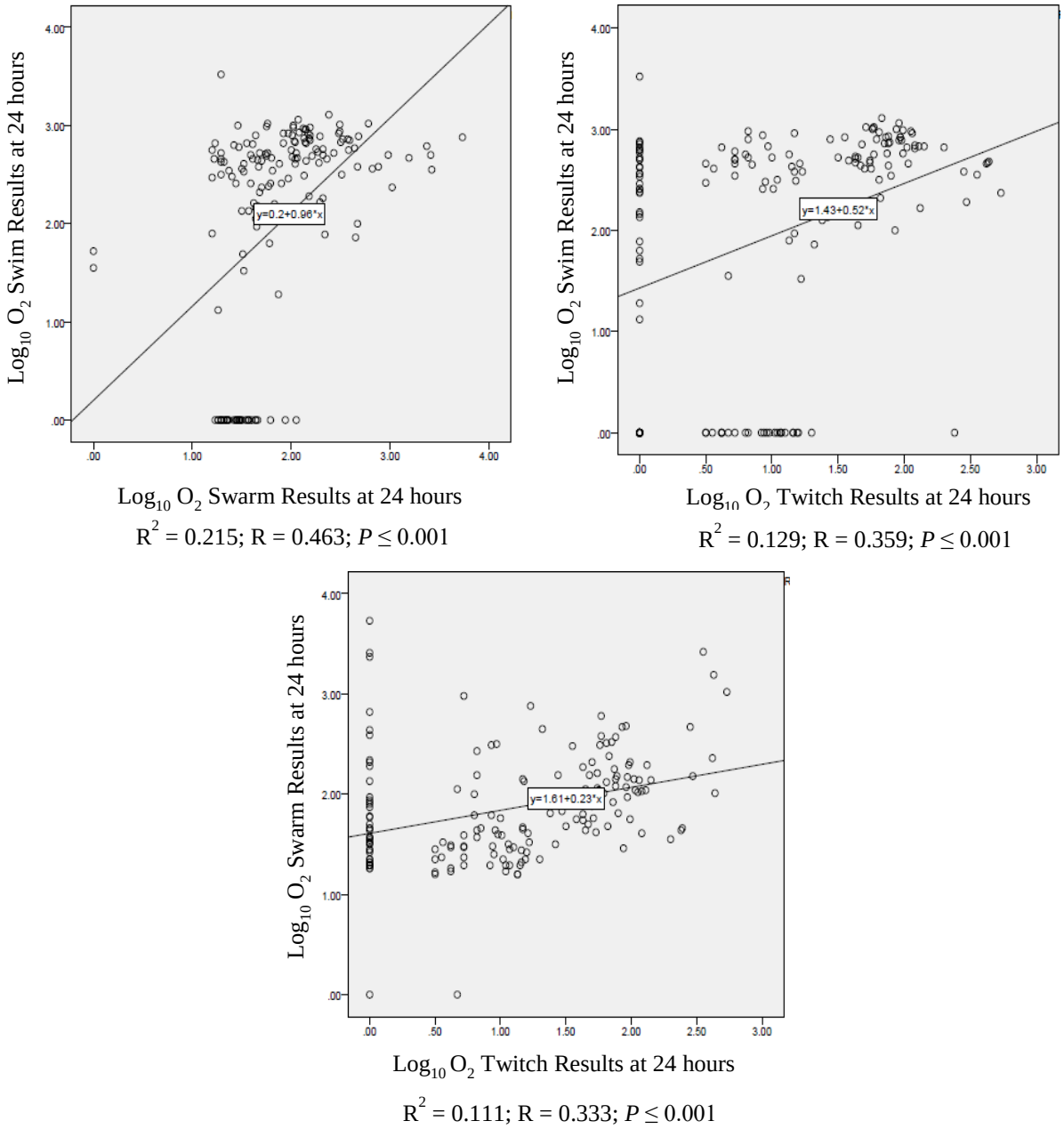
[†] Due to small numbers and as the focus of this study was on the prevalent shared strains identified within the local TPCH CF population, these strains were not included in analysis.

[a,b,c,d,e] Values within columns showing a letter in common were significantly different ($P \leq 0.05$) following Bonferroni correction.

[y,z] Values within columns showing a letter in common were significantly different ($P \leq 0.05$) following Bonferroni correction.

4.4.2.7 Correlation between motilities

The analysis showed that there was a positive correlation when comparing results generated from all isolates for each motility tested under all atmospheric conditions. As an example, there was a positive correlation between swim with swarm motilities under aerobic conditions (R = 0.463; $P < 0.001$), yet only 22% of the variation in swim results was explained by the swarm motility. Similar correlations were seen when comparing swim with twitch and swarm with twitch under aerobic, microaerophilic and anaerobic conditions (Figure 4.3, Appendix 3.8.



$R^2 = 0.215$; R = 0.463; $P \leq 0.001$

$R^2 = 0.129$; R = 0.359; $P \leq 0.001$

$R^2 = 0.111$; R = 0.333; $P \leq 0.001$

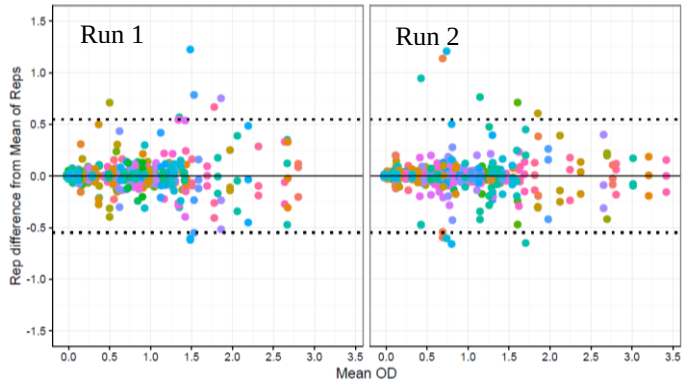
Figure 4.3 Pearson Correlation coefficient results of log transformed data comparing swim, swarm and twitch results following 24-hours incubation in aerobic conditions.

4.4.3 Microtitre plate based assay

4.4.3.1 Reproducibility of microtitre plate based assay

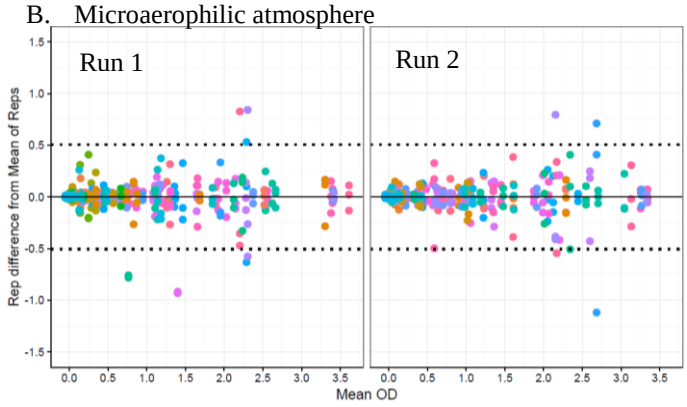
The adhesion assay demonstrated highly reproducible results when both within and between run variability were analysed. Overall, results obtained from tests performed under aerobic (ICC: 0.972 [95% CI: 0.966 to 0.977], $P < 0.001$), microaerophilic (ICC: 0.986 [95% CI: 0.984 to 0.989], $P < 0.001$) and anaerobic (ICC: 0.986 [95% CI: 0.983 to 0.989], $P < 0.001$) conditions determined that agreement measures for this assay were excellent (Figure 4.4, Appendix 3.9). Furthermore, high levels of agreement were also observed within groups when niche and atmospheric conditions were analysed.

A. Aerobic atmosphere



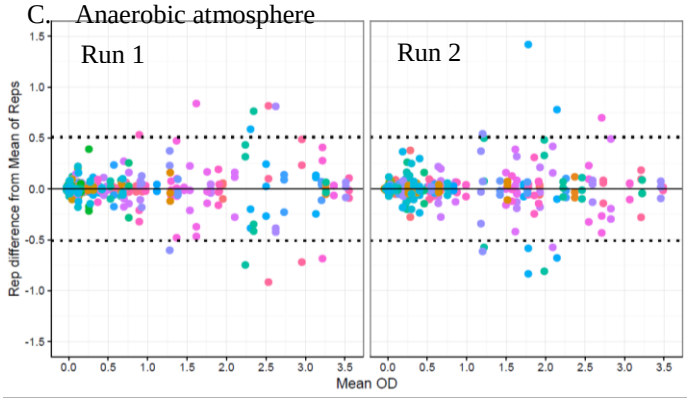
A.
Run 1 ICC: 0.970 (95% CI: 0.961 to 0.977), $P < 0.001$
Run 2 ICC: 0.974 (95% CI: 0.966 to 0.980), $P < 0.001$

B. Microaerophilic atmosphere



B.
Run 1 ICC: 0.982 (95% CI: 0.977 to 0.986), $P < 0.001$
Run 2 ICC: 0.991 (95% CI: 0.988 to 0.993), $P < 0.001$

C. Anaerobic atmosphere



C.
Run 1 ICC: 0.987 (95% CI: 0.983 to 0.990), $P < 0.001$
Run 2 ICC: 0.985 (95% CI: 0.981 to 0.989), $P < 0.001$

Figure 4.4 Reproducibility assessment of the microtitre plate based assay under a) aerobic, b) microaerophilic and c) anaerobic conditions using a generalised Bland-Altman Plot.

Bounds for this plot are ISO-defined reproducibility limits, defined as ±1.96 SD. Each isolate, tested in triplicate, at two different time points (Run 1 and Run 2) is represented by three dots of an individual colour.

4.4.3.2 Enhanced adhesion of non-CF clinical and environmental isolates

Isolates from people with CF demonstrated reduced adhesion under each atmospheric condition when compared with isolates collected from the environment, animal and non-CF human sources. Apart from the comparison between CF and animal isolates under aerobic conditions (P = 0.068) the CF isolates displayed significantly less adhesion for all other assays. When environmental isolates where compared to those collected from animal and non-CF infections, only adhesion tested in aerobic conditions resulted in a significant difference (P = 0.030) (Table 4.6, Panel A).

4.4.3.3 Adhesion characteristics of isolates obtained from people with CF

When the non-shared CF strains were compared to the AUST-02 and AUST-06 strains these isolates displayed enhanced adhesion capabilities. Both shared strains (AUST-02 and AUST-06) demonstrated significantly reduced adhesion capabilities compared to environmental, animal and non-CF isolates, under each atmospheric condition. Finally, no difference was noted when AUST-02 and AUST-06 were compared with both displaying poor adhesion characteristics (Table 4.6, Panel B).

4.4.3.4 Reduced adhesion when tested anaerobically

Apart from animal isolates and the AUST-06 strains, for which there was no change, all other isolate groups demonstrated poorer adhesion capabilities when tested anaerobically, compared to aerobic conditions. However, these results only reached significance for isolates collected from people with CF (P = 0.001), including both the non-shared strains (P = 0.045) and AUST-02 (P = 0.004) (Table 4.6, Appendix 3.6).

Table 4.6 Microtitre plate-based assays tested on *Pseudomonas aeruginosa* isolates collected from human, animal and environmental sources, conducted under different atmospheric conditions, n (%).

Ecological niche or strain	Adhesion-Positive		
	Aerobic	Microaerophilic	Anaerobic
All isolates [§]			
Environmental (n = 34)	34 (100.0) [ac]	28 (82.4) [a]	30 (88.2) [a]
Animal (n = 14)	10 (71.4) [c]	11 (78.6) [b]	10 (71.4) [b]
Non-CF (n = 20)	17 (85.0) [b]	14 (70.0) [c]	15 (75.0) [c]
Cystic Fibrosis (n = 99)	41 (41.1) [ab]	25 (25.3) [abc]	18 (18.2) [abc]
Cystic fibrosis isolates [#]			
Non-Shared Strains (n = 33) [*]	24 (72.7) [yz]	19 (57.6) [y]	15 (45.5) [y]
AUST-02 (n = 50)	15 (30.0) [y]	5 (10.0) [y]	3 (6.0) [y]
AUST-06 (n = 6)	0 (0.0) [z]	0 (0.0)	0 (0.0)
AUST-01 (n = 6) [†]	1 (16.7)	0 (0.0)	0 (0.0)
LES (n = 4) [†]	1 (25.0)	1 (25.0)	0 (0.0)

[§] Bonferroni correction adjusted for 6-way comparison.

[#] Bonferroni correction adjusted for 3-way comparison (Non-Shared, AUST-02 and AUST-06).

[*] Strains found only in individual patients or < 10 of patients in attendance at TPCH.

[†] Due to small numbers and as the focus of this study was on the prevalent shared strains identified within the local TPCH CF population, these strains were not included in analysis.

[a,b,c] Values within columns showing a letter in common were significantly different ($P \leq 0.05$) following Bonferroni correction.

[y,z] Values within columns showing a letter in common were significantly different ($P \leq 0.05$) following Bonferroni correction.

4.4.3.5 Correlation between adhesion and motility assays results across all atmospheric conditions

Positive correlations between the three motilities and adhesion under all atmospheric conditions demonstrated that there was a direct relationship for swimming (R = 0.390; $P < 0.001$), swarming (R = 0.464; $P < 0.001$) and twitching (R = 0.447; $P < 0.001$) when compared to adhesion (Appendix 3.10).

4.4.4 Genotypically indistinguishable strains found across all niches

Five distinct MLST sequence types (ST-155, -179, -253, -266, -381) were each isolated from specimens collected from the natural environment, animal, non-CF human infections and people with CF (Isolates n = 20). Overall, regardless of origin, these isolates behaved more similarly to those isolated from non-CF settings (environment, animal and non-CF), than those isolated from people with CF. Collectively these isolates displayed high rates of motility and adhesion. As described in Table 4.11, 50% of these isolates were non-pigmented, only one isolate displayed a mucoid phenotype, and under aerobic conditions 95%, 75% and 65% of all isolates could swim, swarm and twitch, respectively. Overall, 85% of isolates showed positive adhesion in the microtitre plate based assay (Table 4.7).

Table 4.7 Phenotypic characteristics of the 20 isolates possessing 5 common MLST types isolated from each of the niches, n (%).

Ecological niche			Swim-Positive			Swarm-Positive			Twitch-Positive			Adhesion-Positive		
	Mucoid	Pigment	O_2	MA	AnO_2	O_2	MA	AnO_2	O_2	MA	AnO_2	O_2	MA	AnO_2
Environment	3 (60)	0 (0)	5 (100)	5 (100)	4 (80)	4 (80)	2 (40)	4 (80)	4 (80)	5 (100)	1 (20)	5 (100)	5 (100)	5 (100)
Animal	2 (40)	1 (20)	5 (100)	5 (100)	3 (60)	3 (60)	3 (60)	2 (40)	3 (60)	3 (60)	1 (20)	3 (60)	4 (80)	4 (80)
Non-CF	1 (20)	0 (0)	5 (100)	5 (100)	4 (80)	4 (80)	1 (20)	2 (40)	2 (40)	2 (40)	1 (20)	5 (100)	4 (80)	5 (100)
CF	4 (80)	0 (0)	3 [*] (60)	4 [*] (80)	4 (80)	4 (80)	2 (40)	1 (20)	4 (80)	5 (100)	0 (0)	4 (80)	4 (80)	3 (60)
Total	10 (50)	1 (5)	18 [†] (95)	19 [†] (100)	15 (75)	15 (75)	8 (40)	9 (45)	13 (65)	15 (75)	3 (15)	17 (85)	17 (85)	17 (85)

[*] Total n = 4, one isolate showed an uninterpretable result

[†] Total n = 19

4.5 Discussion

Isolates obtained from a range of clinical settings, including CF, non-CF infections, animal sources and the environment all underwent testing using a range of agar and microtitre based assays in various atmospheric gradients to assess motility and adhesion capabilities. Compared to isolates obtained from the natural environment, isolates cultured from people with CF showed substantially reduced motility and adherence. These phenotypic traits were most pronounced in isolates belonging to shared CF strains and provide further evidence of the highly adapted nature of these genotypes in the CF airway.

An Australian study which reported the high prevalence of numerous genetically indistinguishable strains of *P. aeruginosa* within the national CF population, demonstrated that the presence of these strains was detrimental to the long-term health of patients (Kidd *et al.* 2013). Furthermore, a systematic survey of the local environment and non-CF clinical infections failed to isolate any of the predominant shared Australian CF strains (Kidd *et al.* 2012), suggesting niche adaptation of these strains to the CF airways. Therefore, it has been hypothesised that traits associated with persistence and establishment of infection, such as the production of alginate, the development of a mucoid morphology, the production of siderophores and motility mediated by cell surface organelles, may be a contributing factor to the niche adaptation seen amongst these strains (Folkesson *et al.* 2012; Sousa and Pereira 2014; Winstanley *et al.* 2016). In addition, it is surmised that strains isolated from other sources, with varied genotypes, would not possess mechanisms essential for supporting long term colonisation.

Interestingly, observations from these assays demonstrated both concordant and discordant findings when compared to existing reports in the scientific literature. However, care needs to be taken when interpreting and comparing results across studies, due to differences in experimental design and isolate selection. The key findings from this study are: i) the significant reduction, compared to the reference strain PAO1, of all phenotypic characteristics seen in isolates from people with CF, especially the shared strain AUST-02, ii) enhanced motility and adherence capabilities displayed by isolates collected from the environment, iii) greater similarities between the non-CF isolates, collected from human and animal infections, with the environmental isolates rather than the CF clinical isolates, iv) the reduction in motility and adherence for the majority of isolates, regardless of acquisition source, when tested in anaerobic conditions, and v) the influence of genotype rather than infection status (chronic or intermittent) or origin on phenotypes studied here. Overall, compared to existing data these results demonstrate how different conditions, protocol design and isolate selection influence results.

The CF isolates tested in this study were predominantly isolated from adults with a longstanding chronic *P. aeruginosa* infection (Kidd *et al.* 2013); however, despite this, a clear distinction between the ability to be motile was evident between the non-shared CF strains and the shared strain AUST-02. More specifically, it was observed that the genotypically heterogeneous CF strains displayed a range of different motility traits, demonstrating greater similarities with the isolates from different clinical and environmental niches compared to the AUST-02 strains. Interestingly, there were no differences noted when these non-shared strains were compared to the other highly prevalent shared strain, AUST-06; although these data are difficult to interpret due to a relatively small sample size. These data suggest that rather than being solely associated with chronicity, the genes which regulate motility may be absent or non-functional in the AUST-02 strain. Furthermore, AUST-02 has only been isolated from the CF airway and to date; there has been no evidence of colonisation in any other clinical or environmental setting. Together these results, absent motility and the lack of an environmental reservoir, suggest a very high level of niche adaptation by AUST-02 to the CF airway.

In contrast to this study, genotyping results on the isolates undergoing testing have not been reported for much of the published data. One study which did base isolate selection on genotyping results was the study conducted by Jeukens and colleagues. This study focused on the Liverpool epidemic strain (LES), a highly prevalent strain most commonly isolated from patients residing in the United Kingdom and Canada (Aaron *et al.* 2010; Scott and Pitt 2004). Results from this study demonstrated that compared to a reference strain and isogenically similar strains, each of LES isolates displayed either a reduced or absent phenotype (Jeukens *et al.* 2014). However, rather than selecting isolates based on strain type a large proportion of existing work undertaken in this area has been conducted on isolates stratified according to disease severity. These studies agree that isolates obtained from chronic infection sources have reduced motility phenotypes compared to isolates from early infection (Burke *et al.* 1991; Cullen *et al.* 2015; Jeukens *et al.* 2014; Lee *et al.* 2005; Mahenthiralingam *et al.* 1994; Manos *et al.* 2013; Mayer-Hamblett *et al.* 2014). It is difficult to determine if results from these studies are biased based on genotypes as only a few include these data. However, for those studies which do comment, isolates with unique sequence types comprise the majority of strains (Head and Yu 2004; Manos *et al.* 2013). Rather than demonstrating a loss of motility based on infection status, I have shown that isolates from chronically infected patients, with a unique sequence type more closely resembled isolates from other clinical settings with moderate to high levels of positive motility reported.

Similar to this study, a number of published studies have included non-CF and/or environmental isolates for comparison (Cullen *et al.* 2015; Head and Yu 2004; Jeukens *et al.* 2014;

Mahenthiralingam *et al.* 1994; O'May *et al.* 2006). Results from the current work are concordant with these earlier studies; both demonstrating enhanced motility among isolates arising from ecological niches outside of CF.

To date the majority of studies previously reported have been conducted under aerobic atmospheric conditions with very few reporting motility characteristics under a reduced oxygen environment. Due to CF-specific increases in epithelial oxygen consumption, linked to increased airway surface liquid volume absorption and mucus stasis, varied oxygen gradients are present within mucus residing on epithelial surfaces (Worlitzsch *et al.* 2002). To reflect these conditions, all aspects of this study were performed in aerobic, microaerophilic and anaerobic conditions. Overall, this study demonstrated that swim and twitch motility for the majority of isolates was reduced when assessed in microaerophilic and anaerobic conditions alike; although these results rarely achieved statistical significance. Interestingly, swarming was not negatively correlated with anaerobic conditions to the same degree as swim and twitch were. When assessing the impact of anaerobic conditions on all isolates, there was only a 3% decline observed for swarming motility compared to a 23% and 28% reduction in swimming and twitching, respectively. As this result may be biased due to the large number of swarm negative AUST-02 strains, adjustment for all CF isolates resulted in an increase from 37% to 68% for swarming in anaerobic conditions. Isolates from the environmental demonstrated an enhanced capability to swarm under anaerobic conditions and this may reflect the diverse settings these organisms are collected from. O'May and colleagues, performed similar analyses, comparing the motility of CF and non-CF isolates in aerobic and anaerobic conditions. This study reported a marked decrease in each motility when tested anaerobically, regardless of clinical source. More specifically, 100% of CF isolates displayed smaller motility zones when swarm was tested anaerobically compared to aerobically, whereas, only 50% of the non-CF isolates demonstrated a reduced swarm zone (O'May *et al.* 2006). Results from both studies demonstrate that atmospheric conditions do impact motility and this effect is more pronounced for the clinical isolates obtained from people with CF. Therefore, data reporting motility tested only in aerobic conditions should be interpreted with care as outcomes may vary when assessed under different experimental conditions.

Similar to the motility results, a significant reduction in the adhesion capabilities was noted for the CF isolates, in particular the shared CF strains. These studies were conducted using a polystyrene microtitre plate in aerobic, microaerophilic and anaerobic conditions. Strong adhesion to the plastic surface was observed for the environmental, animal and non-CF isolates. As mentioned previously the isolates obtained from people with CF were predominantly associated with chronic infections. Unrelated to infection status, the non-shared CF strains displayed greater similarities regarding

adhesion with the isolates collected from the environment, animal and human clinical infections than with the shared CF strains. Overall, the shared strains displayed virtually no adherence to the plastic substrate. These results are of interest, as both shared and non-shared CF strains, causing chronic infections displayed very different abilities to adhere in this assay. Assessing these results, in isolation, suggests that this specific trait may not be currently required to facilitate the establishment and persistence of an infection and following adaptation to the CF airway adherence is selected against.

Due to large variations between methodologies and experimental conditions which exist within the published literature, it is difficult to compare and contrast the adhesion capabilities of *P. aeruginosa* isolates (Azeredo *et al.* 2017). Regardless of these differences, the consensus among the literature, demonstrates that the CF strains display poorer adhesion compared to non-CF strains (Cullen *et al.* 2015; Head and Yu 2004; Jeukens *et al.* 2014; O'May *et al.* 2006). However, there does appear to be greater heterogeneity seen in the published literature when comparing shared and non-shared CF strains to each other and to non-CF isolates. There are reports of both enhanced and subdued adherence among these comparisons (Cullen *et al.* 2015; Head and Yu 2004; Jeukens *et al.* 2014; O'May *et al.* 2006). Again, similar to the published data on motility, these results are also highly influenced by isolates obtained from sources where *P. aeruginosa* has been associated with a chronic infection.

As observed for motility when tested anaerobically, bacterial adhesion was also reduced when tested in oxygen limiting conditions. Only a slight reduction in adhesion was observed for environmental and non-CF isolates and no change in adherence capabilities were reported for animal isolates. However, there was a statistical significance reported when the CF isolates were tested anaerobically, including both the non-shared and AUST-02 strains alike. These data contrast earlier studies which demonstrated that rather than having a negative impact on adherence, the lack of oxygen can actually enhance this phenotype (O'May *et al.* 2006; Yoon *et al.* 2002). Again, the degree to which this result is observed appears to be dependent upon methodology and strain. While, consistently poorer biofilm producers than PAO1, CF and non-CF strains have all demonstrated improved adherence under anaerobic conditions, although this increase is often modest (O'May *et al.* 2006). By utilising a number of static adhesion assays designed to better replicate the growth mode of *P. aeruginosa* in mucus plugs, Yoon and colleagues, were able to clearly demonstrated that not only could *P. aeruginosa* form biofilms and adhere to surfaces under anaerobic conditions, this phenotype was enhanced when compared to growth in aerobic conditions. Nevertheless, these results must be interpreted with caution as only the PAO1 strain underwent testing (Yoon *et al.* 2002). Regardless,

these studies, together with the current work demonstrate that while atmospheric conditions can influence the degree of adhesion, it does not prevent it from occurring.

Analysis in this chapter also revealed a strong correlation between motility and adhesion capabilities. Specifically, these data showed that those isolates which were able to swim were also able to swarm and twitch. Furthermore, isolates which displayed enhance motility also demonstrated an enhanced capacity for adherence in the microtitre plate. Similar to other phenotypic traits, contrasting evidence determining an association between motility and the ability to form biofilms has also been reported. Jeukens and colleagues showed that isolates lacking motility were unable to adhere to the surface of the microtitre plate, whereas motile isolates produced thicker biofilms. This observation was independent of twitching motility (Jeukens *et al.* 2014). Results from studies assessing the motility and adhesion capabilities of a number of non-CF clinically significant isolates demonstrated enhanced motility and biofilm formation for all isolates (Murray *et al.* 2010; O'May *et al.* 2006). In addition, Murray and colleagues reported an inverse relationship between the ability to swim and twitch compared to the development of biofilm under static conditions (Murray *et al.* 2010).

Several studies have observed that as the infection duration increases, CF isolates have a tendency to become non-motile. However a similar reduction in adherence has not been reported for all studies (Cullen *et al.* 2015; Head and Yu 2004; Lee *et al.* 2005). Lee and colleagues demonstrated a corresponding reduction in both motility and adhesion using the microtitre plate-based assay (Lee *et al.* 2005). These results were replicated using the flow chamber, demonstrating that as a chronic infection developed the corresponding reduction in motility lead to decreased attachment of cells to the coverslip and a reduction in microcolony development. As this assessment was qualitative, the degree of reduction could not be determined. Furthermore, this study reported that twitching motility, mediated by functional type IV pili, resulted in uniform biofilms with an even homogeneous layer of cells at the coverslip; whereas isolates that were unable to twitch produced thin biofilms with an uneven, heterogeneous appearance (Lee *et al.* 2005). However, Head and Yu concluded that there was no association between motility and biofilm formation using a plate-based assay. They demonstrated that a number of isolates with increased motility had reduced biofilm formation and conversely a number produced thick biofilms. This study determined that although all isolates possessed flagella, is not a fundamental mechanism required for biofilm formation. Furthermore, they determined that isolates lacking the ability to twitch produced high levels of biofilm (Head and Yu 2004).

To my knowledge, the current study is the first to assess bacterial motility and adherence among genotypically indistinguishable strains isolated from different ecological niches. Twenty isolates comprising five distinct MLST sequence types (ST-155, -179, -253, -266, -381) cultured from four different ecological niches were included for testing. These particular strains have previously been isolated during environmental surveillance, clinical diagnosis and epidemiological surveys (Kidd *et al.* 2015; Kidd *et al.* 2012; Ranganathan *et al.* 2013). Irrespective of acquisition source, these strains displayed phenotypic characteristics similar to other isolates obtained from non-CF settings (i.e. the environment, animals or non-CF patient sources). Retention of motility and adhesion capabilities reflect the diversity and highly adaptable nature of these strains. Longitudinal analysis of phenotypic characteristics during the establishment of a chronic infection in a person with CF would be needed to determine, if over time and due to ongoing exposure to host factors and antimicrobial substances, whether these strains adapt to the CF airways in a manner similar to that seen with the AUST-02 strain.

During the establishment of an infection, *P. aeruginosa* uses motility and adhesion to colonise and develop bacterial community structures known as biofilms. *P. aeruginosa* possess cell surface organelles, pili and flagella which are responsible for cellular movement (swim, swarm and twitch) and the adhesion of the bacterial cells to each other, biotic or abiotic surfaces. These cellular mechanisms are complex and are regulated by a number of interconnecting pathways, genetic mutations and cell-density-dependent communication (Alhede *et al.* 2014; Alhede *et al.* 2011; Bjarnsholt 2013; Costerton *et al.* 1995; Davey and O'Toole G 2000; Winstanley *et al.* 2016). The development of biofilms within the lungs and a corresponding decline in cellular motility are often cited as indication that a chronic infection has ensued. Over recent years a number of methodologies have been developed which use surface adhesion to infer biofilm development (Azeredo *et al.* 2017; McBain 2009; Roberts *et al.* 2015). Static growth in the microtitre plate based assay is the mostly commonly used experimental design and allows for the quantification of cells displaying adhesion to be easily measured. This method is high throughput and easy to use and interpret (Azeredo *et al.* 2017; Roberts *et al.* 2015). From this study, results would suggest that the CF isolates, in particular the AUST-02 strain, which are non-motile and almost universally non-adherent would not be able to adhere to surfaces within the airways and therefore may be unable to establish robust biofilms. As this strain is most often isolated from people with CF and chronic infection these data appear to be at odds with earlier work which suggest that as organisms adapt to the CF airway there is a progression from planktonic to biofilm-associated growth.

A big challenge facing this study was the isolate selection. While a wide variety of unique and shared genotypes, isolated from a vast range of clinical and environmental settings were chosen, a large proportion of the isolates tested belonged to the AUST-02 genotype. The AUST-02 strain was a particular focus of this study due to: i) its high prevalence within the Queensland CF population, ii) the absence of an environment reservoir, iii) its association with patient to patient transmission, and iv) the likelihood that it is highly adapted to the CF airway. By including so many of these strains for analysis it could be argued that this is repetition of a single strain and therefore multiple testing is potentially superfluous and creates bias in the interpretation of the results. From the stark results obtained, distinctively different phenotype is still evident for all of the CF isolates, regardless of the inclusion of the AUST-02 strains or not. Furthermore, works undertaken by colleagues assessing diversity within the AUST-02 strain have identified sub-types, each with different prevalence, phenotypic traits and antimicrobial profiles (Sherrard *et al.* 2017; Tai *et al.* 2015). Although sub-type analysis was not undertaken on these isolates, phenotypic results from these tests have identified a small number of strains with different characteristics, however overall the AUST-02 demonstrated an absence of motility and adhesion traits. Therefore, testing a large number of the AUST-02 strains was warranted.

Despite high levels of reproducibility reported among the various methodologies employed throughout these analyses, this study had a number of limitations specific to assay design. Firstly, the approach of qualitatively reporting the colonial morphology, rather than assessing these traits through quantitative assessment may have underestimated the prevalence of certain phenotypes within the isolates tested. Secondly, rather than using established knockout mutants, known to display or suppress the desired phenotypic characteristic, as reference strains, all test isolates were compared to the *P. aeruginosa* strain, PAO1. Although PAO1 is often cited as a reference strain throughout the published literature, this strain may not be the most appropriate choice for the comparison of test isolates. PAO1 has been utilised as a reference strain for more than 60 years, and is now considered a laboratory strain, displaying distinct characteristics from clinical strains. Therefore, using this strain as the reference to which all other isolates are categorised may result in the misinterpretation of phenotypic characteristics as displayed by the test isolates. Thirdly, results generated using the static biofilm methodology was not confirmed using a dynamic system, such as the flow chamber, for a more thorough comparison. Although this additional testing would have been time consuming and expensive there is little published data to suggest correlation between the two assays. Fourthly, due to the small number of AUST-06 strains included for testing, these results must be interpreted with care. At the time of commencing this study the AUST-02 was the predominant shared strain identified at the TPCH Adult CF Clinic and therefore was the focus of the study. Finally, no genetic analysis

was undertaken on these isolates. By omitting analysis such as whole genome sequencing to identify mutations associated with the presence or absence of phenotypic characteristics, and by not confirming the effect of these mutations on protein functionality through expression assays, it is difficult to determine if the *in vitro* phenotypes reported in these studies will be an accurate representation of characteristics which may be displayed *in vivo*. Furthermore, the effect of selective pressures such as host immune factors and antibiotic exposure may also contribute to discrepancies between the *in vitro* and *in vivo* results. Therefore, these factors need to be considered when interpreting results generated from these analyses.

Overall, these data also provide compelling evidence that exposure to different atmospheric conditions plays an important role in bacterial motility and adherence, and have important implications for our understanding biofilm biology in CF airways and highlight the need for cautious interpretation of earlier work in this field that primarily focused on aerobic experimental conditions. Furthermore, I demonstrate that *P. aeruginosa* isolated from the airways of chronically infected people with CF show reduced motility and adhesion as compared to isolates arising from other ecological settings. These phenotypic characteristics were most evident among shared CF strains, in particular the AUST-02 strain, and thus provide further evidence that these genotypes are highly adapted to the CF airway. Due to the complexity of airway infection process in CF, including the role of host factors and bacterial virulence, it is difficult to directly relate results from phenotypic analyses to broader health outcomes, in particular how reduced motility and altered adhesion capabilities impact on lung function. Future studies on a sub-set of these isolates, displaying distinct phenotypic characteristics, will be undertaken to elucidate additional adaptive mechanisms. A dynamic flow cell apparatus will be utilised to determine the influence these traits have on the development of sessile community formations known as biofilms. Furthermore, whole genome sequencing will be performed to provide further mechanistic insights to the biofilm, motility and adhesion characteristics of both environmental and significant clinical strains of *P. aeruginosa*.

Chapter 5: Biofilm development of *Pseudomonas aeruginosa*

5.1 Abstract

Background: The bacterium *P. aeruginosa* undergoes a number of phenotypic changes during the establishment of chronic infection in people with cystic fibrosis (CF). Previous phenotypic analysis of a large isolate cohort demonstrated that isolates from acute non-CF infections and the environment were highly motile and displayed enhanced adhesion in a microtitre plate-based assay. In contrast, isolates from CF patients, including the prevalent Australian shared strain, AUST-02, were non-motile and almost universally non-adherent. By studying a subset of isolates displaying either enhanced or absent phenotypic characteristics, this study aimed to determine if motility and adhesion are required for the development of micro-colonies in mature biofilms.

Methods: Eighteen isolates (AUST-02 n = 12, environment n = 6) were grown within a flow cell system used to visualise the maturation dynamics of in vitro biofilm structures. Following incubation at 30°C for 72-hours a live/dead stain was injected into the flow-cell chamber. Three dimensional images were captured with confocal microscopy and analysed by Comstat software. Effluent was serially diluted and quantified to determine bacterial dispersal levels. Comparative analysis of six environmental and 8 individual patient AUST-02 isolates was undertaken. In addition, six longitudinally collected AUST-02 isolates (including two isolates included in the comparative analysis) arising from two patients was assessed for changes in biofilm parameters over time. Mixed effects models were used to compare relevant biofilm parameters, with a P value of <0.05 considered significant.

Results: Analysis comparing biofilm development of eight AUST-02 isolates and six environmental isolates demonstrated that the AUST-02 strains formed thicker biofilms ($P < 0.001$) with increased biomass ($P = 0.012$) and greater surface area coverage ($P = 0.002$) compared to the environmental isolates. Furthermore, significantly more organisms were cultured from the effluent of the AUST-02 strain flow cells (3.0 x 108 versus 6.2 x 107; $P = 0.008$), suggesting enhanced dispersal. Six longitudinally collected isolates (including two isolates included in the comparative analysis) from two patients demonstrated that there was no change to any of the biofilm parameters overtime (biomass $P = 0.089$, maximum thickness $P = 0.461$, surface area $P = 0.164$).

Conclusions: AUST-02 form robust mature biofilm structures despite apparent poor motility and surface adhesion. These results vary from published work, highlighting the variability and diversity of *P. aeruginosa*.

5.2 Introduction

Bacterial biofilms represent an adaptive growth mode which allows for the colonisation and survival of microorganisms under extreme environmental conditions (Hall-Stoodley *et al.* 2004; Hall-Stoodley and Stoodley 2009; Hall-Stoodley and Stoodley 2005). Biofilms develop when there is an influx of motile planktonic cells adhering to either a surface and or to each other, followed by the production of extracellular polymeric substances (EPS) and the formation of three dimensional (3D) cellular structures (Hall-Stoodley *et al.* 2004; Wilkins *et al.* 2014). EPS, comprising of extracellular DNA, proteins and polysaccharides is integral to the structural formation of biofilms.

In contrast to bacterial cells living within the protection of a biofilm structure, planktonic cells are exposed to varied environmental conditions, host immune factors and antimicrobial substances. These cells are distinct from their biofilm counterparts and have been shown to possess a range of characteristics, not displayed by isolates within biofilms. During the transition from a planktonic mode of growth, bacterial cells undergo a range of adaptations promoting biofilm formation, which increases the bacterium's ability to withstand eradication via the host immune system and antimicrobial resistance (Alhede *et al.* 2009; Chua *et al.* 2015; Colvin *et al.* 2011; Leid *et al.* 2005; Roberts *et al.* 2015; Stoodley *et al.* 2002; Winstanley *et al.* 2016). From a clinical perspective, the formation of biofilms is particularly relevant as they have been associated with a number of infective processes including catheter colonisation, implanted device, cutaneous and chronic respiratory infection (Bjarnsholt 2013).

Pseudomonas aeruginosa is an environmental bacterium which is capable of existing in both planktonic and biofilm growth states. This opportunistic pathogen most commonly causes infections in immunocompromised hosts and is frequently associated with multidrug resistance (Llanes *et al.* 2013; Silby *et al.* 2011). *P. aeruginosa* is the most prevalent pathogen isolated from the respiratory tract of people with cystic fibrosis (CF) and is the leading cause of morbidity and mortality among this patient group (Emerson *et al.* 2002; Sanders *et al.* 2014). As a result of increased resistance to anti-pseudomonal antibiotics, the establishment of chronic infection and biofilm growth, eradication of *P. aeruginosa* from the airways is extremely difficult (Vidya *et al.* 2016). Because of these unique properties and owing to its significant clinical relevance, *P. aeruginosa* is an excellent model to assess biofilm growth.

Phenotypic characterisation of *P. aeruginosa* isolated from people with CF has demonstrated the importance of functional pili and flagella during the attachment process and subsequent formation of biofilm structures. Non-motile isolates display poor adhesion and reduced structural formation of the

biofilms compared to isolates demonstrating enhanced motility (Chiang and Burrows 2003; Jeukens *et al.* 2014; Lee *et al.* 2005; O'Toole and Kolter 1998). However, not all studies demonstrate a strong correlation between motility, adhesion and biofilm formation. When assessing non-CF clinical isolates, Murray and colleagues demonstrated that while swim and twitch motility mediated by flagella and pili were required for biofilm development, the lack of a swarming motility phenotype did not impede biofilm development (Murray *et al.* 2010). Likewise, Head and Yu describing the characteristics of CF, non-CF and environmental isolates reported that there was no correlation between biofilm formation and motility (Head and Yu 2004). In addition, duration of infection and strain type appear to influence the phenotypic characteristics, with a reduction in motility and biofilm formation noted for persistent and chronic isolates, as well as, isolates belonging to some prevalent shared CF strains (Duong *et al.* 2015; Jeukens *et al.* 2014; Lee *et al.* 2005; Manos *et al.* 2013; Mayer-Hamblett *et al.* 2014).

There are several different *in vitro* models that can be used to assess bacterial cellular adherence and biofilm formation (Azeredo *et al.* 2017; Crusz *et al.* 2012; McBain 2009; Roberts *et al.* 2015); therefore, it is feasible that variations in the methods used may also play an important role in the lack of concordance between studies. Owing to their high throughput nature, microtitre plate-based models offer a rapid means of screening large numbers of isolates to determine cellular adhesion and short-term biofilm forming capacity using a range of different experimental conditions. However, due to nutrient depletion these assays cannot be performed over extended time periods. Furthermore, they provide no information on the structure of the biofilms generated. In contrast, the flow cell method allows for the maturation of biofilms within a dynamic system. This apparatus utilises an intricate system of tubing and a pump to allow for the continual supply of nutrients and the removal of planktonic cells and effluent over the duration of the experiment. While this methodology allows for the microscopic visualisation of architectural biofilm features and assessment of bacterial biofilm dispersal, it too has several limitations. Temperature and pressure changes within the system can promote the formation of air bubbles which can impact on the growth, and even destroy the biofilm. Bacterial overgrowth of isolates can impede biofilm maturation. System assembly is also technically challenging, and time consuming, thus relatively low throughput and individual modifications in addition to the published methodology are required to optimise specific experimental conditions (Azeredo *et al.* 2017; Crusz *et al.* 2012; Heydorn *et al.* 2000).

It is hypothesised that those isolates which demonstrate strong adhesion capabilities in the microtitre assay will also develop biofilm structures within the flow cell model. Furthermore, for the persistent clinical isolates, length of infection will impact biofilm development. Unfortunately, due to a lack of

consensus in the scientific literature regarding methodology, experimental conditions, analysis techniques and bacterial strains, it is difficult to determine the most appropriate protocol to use when determining an isolate's capability to establish a biofilm. Furthermore, there has not been a comparison of non-motile, poorly adherent shared CF strains versus highly motile and adherent isolates obtained from natural environment sources to assess specific bacterial growth characteristics. To address these knowledge gaps, this study aimed to: i) assess the concordance and reproducibility of the static microtitre plate-based biofilm model and the dynamic flow cell assay, ii) assess the *in vitro* biofilm forming capacity and extent of bacterial dispersal among isolates derived from different ecological backgrounds and possessing distinct motility and adherence phenotypes, and iii) determine whether duration of infection impacts biofilm development.

5.3 Methods

5.3.1 Isolate Selection

In Chapter 4 it was demonstrated that across a range of atmospheric conditions, isolates from environmental settings showed a highly motile (i.e. swim, swarm and twitch) and adherent phenotype utilising a microtitre plate-based assay. In contrast, isolates belonging to the AUST-02 shared strain were most frequently non-motile and non-adherent. Based on these data it was hypothesised that the AUST-02 strain would show a reduced capacity to adhere to substratum and form mature biofilm structures in the flow cell assay, whereas environmental organisms would produce long-term biofilms of increased biomass. To this end, six environmental and 12 AUST-02 isolates were selected for analysis in the flow cell system (Table 5.1).

The environmental *P. aeruginosa* isolates were collected in 2009 during a survey of three riverine systems situated in South East Queensland, Australia (Kidd *et al.* 2012). Multilocus sequence typing (MLST) confirmed that all isolates possessed individual sequence types (ST-155, -179, -253 [aka PA14], -266, -381 and -471) which have also been isolated from persons with CF residing in South East Queensland (Kidd *et al.* 2012). The 12 AUST-02 isolates were collected between 2002 and 2013 from sputum samples provided by CF patients participating in one of two cross sectional prevalence studies (Kidd *et al.* 2013; O'Carroll *et al.* 2004). All patients resided in South East Queensland at the time of collection and attended CF clinics at The Prince Charles Hospital (TPCH) or Royal Children's Hospital (RCH), Brisbane. All 12 AUST-02 isolates were confirmed by MLST as ST-775 which is exclusively linked to this prevalent Queensland shared strain (Kidd *et al.* 2011; Kidd *et al.* 2012; *Pseudomonas aeruginosa* PubMLST Database 2012). The AUST-02 isolate collection comprised six isolates each cultured from individual patients, as well as, six longitudinally collected isolates obtained from two chronically infected patients during the years 2002, 2007 and 2013 (Kidd *et al.* 2013) (Table 5.1). Patient 1, a 16-year-old male (at 2002), and Patient 2, a 14-year-old female (at 2002), first acquired *P. aeruginosa* at the ages of 10 and 8 years, respectively. Patient 1 was heterozygous the p.F508del mutation (p.F508del/N130K), whereas Patient 2 was homozygous for p.F508del mutation. Two of the longitudinally collected isolates were used alongside the other six AUST-02 isolates for the broader comparisons with the environmental isolates, and all six were used to assess the association between phenotype and biofilm formation over time. In addition, *P. aeruginosa* strain PAO1 was used as a control isolate for all assays (Holloway 1955).

5.3.2 Sample Collection, Isolate Processing and Phenotypic Analysis

Sample collection and culture, bacterial identification, MLST and phenotyping (i.e. colonial morphology; swim, swarm and twitch motility; microtitre plate-based assay using LB broth) of the 18 test isolates was performed as detailed in Chapter 4 (Sections 4.3.1 – 4.3.7). For the purposes of this study a subset analyses of the phenotypic characteristics was undertaken for all 18 isolates.

5.3.3 Growth curve analysis

Bacterial fitness and generation time was determined for the all isolates, including PAO1 (n = 4, biological replicates). A 50 µL aliquot of an overnight bacterial culture prepared in LB media was diluted into 20 mL of M63 media and aerobically incubated with shaking (37 °C/200 rpm). Density readings and total viable counts enumerated on Mueller-Hinton agar were determined after 0, 4, 8, 12, 16, 24 and 36 hours (McCaughey *et al.* 2013; Sherrard *et al.* 2017).

5.3.4 Extended Microtitre Plate-based Assay Analysis

In order to more closely represent and replicate the conditions within the flow cell, the microtitre plate-based assay was repeated using M63 media (as described in Section 4.3.7.5). Bacterial adhesion was tested in this model on all 18 isolates after 24hours incubation in microaerophilic conditions.

Table 5.1 Isolate selection for *in vitro* flow chamber experiments, (n = 18).

Number	Source	Sample Type	Year of Collection	Additional sample & genotype information	MLST	Mucoid	Pigment
1	Environment	Water	2009	Brisbane River; Urban	155	-	+
2	Environment	Swab; Air/Water Interface	2009	Logan River; Rural	179	-	-
3	Environment	Swab; Air/Water Interface	2009	Brisbane River; PA14; Urban	253	-	-
4	Environment	Water	2009	Brisbane River; Rural	266	-	+
5	Environment	Water	2009	Brisbane River; Urban	381	-	+
6	Environment	Water	2009	Brisbane River; Urban	471	-	+
7	CF (Adult)	Sputum	2007	Shared CF strain; AUST-02	775	-	-
8	CF (Adult)	Sputum	2005	Shared CF strain; AUST-02	775	-	-
9	CF (Adult)	Sputum	2007	Shared CF strain; AUST-02	775	-	-
10	CF (Adult)	Sputum	2008	Shared CF strain; AUST-02	775	-	+
11	CF (Paediatric)	Sputum	2007	Shared CF strain; AUST-02	775	-	+
12	CF (Paediatric)	Sputum	2007	Shared CF strain; AUST-02	775	+	+
13 [a]	CF (Paediatric)	Sputum	2002	Shared CF strain; AUST-02	775	-	-
14 [a,c]	CF (Adult)	Sputum	2007	Shared CF strain; AUST-02	775	-	+
15 [a]	CF (Adult)	Sputum	2013	Shared CF strain; AUST-02	775	-	-
16 [b]	CF (Paediatric)	Sputum	2002	Shared CF strain; AUST-02	775	-	+
17 [b]	CF (Adult)	Sputum	2007	Shared CF strain; AUST-02	775	-	+
18 [b,c]	CF (Adult)	Sputum	2013	Shared CF strain; AUST-02	775	-	+

[a] Longitudinally collected isolates from Patient 1; CF chronically infected with AUST-02.

[b] Longitudinally collected isolates from Patient 2; CF chronically infected with AUST-02.

[c] Included in both environmental and AUST-02 comparison and longitudinal analysis.

5.3.5 Flow Cell Assay

The flow cell system was set up in accordance with the model described by Crusz and colleagues with minor modifications (Crusz *et al.* 2012). Figure 5.1 shows the main components of the system which comprised: i) one litre glass bottles (Schott AG, Mainz, Germany) for housing sterile media to support the growth of the target microorganisms and to act as a waste receptacle; ii) bubble traps (Technical University of Denmark) in which the media flows through prior to reaching the flow cell to facilitate the removal of any bubbles; iii) a constructed flow cell in which the bacteria grow and adhere to the glass substratum allowing for visualisation using a confocal microscope; iv) and a peristaltic pump (Ismatec®, Process Pumps, Boronia, Australia) which drives the media through the system. All components were joined by silicon tubing of various internal diameters (ID) (Dow Corning Australia Pty Ltd, North Ryde, Australia; Saint-Gobain, Pinkenba, Australia) and plastic connectors (Cole-Parmer Instrument Company, Illinois, USA). Owing to the increased forces exerted on the pump tubing, stronger Ismaprene tubing (internal diameter 1.02 mm, Idex Health & Science GmbH, Wertheim, Germany) was used. Excluding the waste bottles, all components of the flow cell system were placed at the same level. Waste bottles were positioned below the apparatus to facilitate media flow and prevent bubble formation.

5.3.5.1 Flow Cell Construction

Flow cells used in this study were manufactured by the Department of Systems Biology, Technical University of Denmark. Each flow cell was machine manufactured from Perspex and consisted of three parallel chambers each of a 40 x 4 x 0.3 mm internal diameter. Glass coverslips (24 x 50 mm; Menzel-Gläser®, Branschweig, Germany), which act as the substratum for bacterial growth, were adhered to the flow cell using silicon glue. Prior to bonding, and to enhance bacterial adhesion, each coverslip was washed for two hours in a solution of two parts 15.7 M HNO_3 (nitric acid) and one part 2 M HCl (hydrochloric acid) (Aqua Regia), rinsed in running water and then stored in 80% (v/v) ethanol (Fischer *et al.* 2008). Upon use each coverslip was briefly flamed and then gently adhered to a thin layer of silicone glue applied along the length of the Perspex ridge running between the flow cell channels. Constructed flow cells were left overnight to allow the silicon glue to set, and then visually inspected to ensure nil leakage, even coverslip adherence and optimal fluid movement.

5.3.5.2 Sterilisation and Washing of the Flow Cell System

Sterilisation of the flow cell system was achieved by pumping one litre of 0.5% (v/v) NaClO (sodium hypochlorite; Labtek Pty. Ltd., Brendale, Australia) through the system for three hours at a speed of 80 mL per hour per channel. The system was then emptied and one litre of sterilised

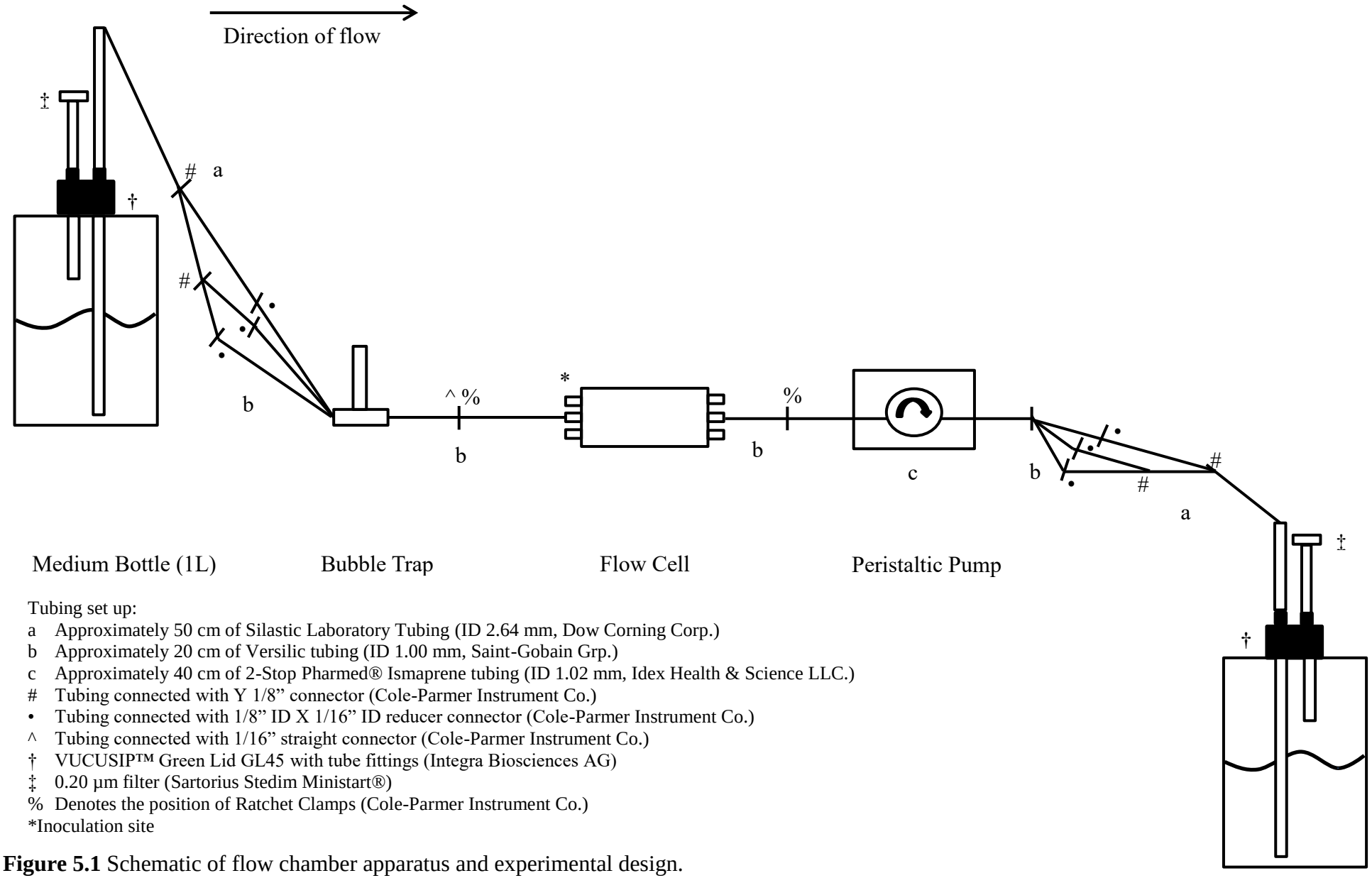
Milli-Q water was run through it for three hours to remove any traces of residual bleach. Flow cell sterilisation was performed both prior to and following each experiment.

5.3.5.3 Media Preparation

Minimal media, M63, was used for all flow cell experiments and initial bacterial suspension preparation. M63 contained 15 mM $(NH_4)_2SO_4$ (ammonium sulfate), 22 mM KH_2PO_4 (potassium dihydrogen phosphate), 40 mM K_2HPO_4 (potassium phosphate dibasic) supplemented with filter sterilised 1 mM $MgSO_4$ (magnesium sulfate), 10 µM $FeCl_3$ (iron III chloride) and 5 mM glucose following autoclave sterilisation.

5.3.5.4 Filling of the Flow Cell System

Following assembly and cleaning of the flow cell system, the M63 media was passed through the system prior to inoculation. Media was introduced into the system at 3.0 mL h^{-1} $channel^{-1}$ and let run through until all tubes were comprehensively filled with media running into the effluent bottle. The pump was then stopped and the caps on the bubble traps removed. Under the influence of gravity the bubble traps filled with media. Once full, the bubble trap caps were replaced and the media flow was resumed at 3.0 mL per hour per channel (50 µL/minute) overnight prior to inoculation.



Tubing set up:

a Approximately 50 cm of Silastic Laboratory Tubing (ID 2.64 mm, Dow Corning Corp.)
b Approximately 20 cm of Versilic tubing (ID 1.00 mm, Saint-Gobain Grp.)
c Approximately 40 cm of 2-Stop Pharmed® Ismaprene tubing (ID 1.02 mm, Idex Health & Science LLC.)
Tubing connected with Y 1/8" connector (Cole-Parmer Instrument Co.)
• Tubing connected with 1/8" ID X 1/16" ID reducer connector (Cole-Parmer Instrument Co.)
^ Tubing connected with 1/16" straight connector (Cole-Parmer Instrument Co.)
† VUCUSIP™ Green Lid GL45 with tube fittings (Integra Biosciences AG)
‡ 0.20 µm filter (Sartorius Stedim Ministart®)
% Denotes the position of Ratchet Clamps (Cole-Parmer Instrument Co.)
*Inoculation site

Figure 5.1 Schematic of flow chamber apparatus and experimental design.

5.3.5.5 Preparation of Bacterial Inoculum

All isolates were tested in triplicate (biological replicates) with 10 mL of M63 broth inoculated with 1 µL of bacteria. Following overnight incubation at 37 °C/200 rpm, the bacterial suspension was adjusted to an optical density of 0.1 nm (OD_{600nm} = 0.1), using sterile M63 media. The control PAO1 strain and was tested on two separate occasions (technical replicates) with a total of four biological replicates.

5.3.5.6 Flow Cell Inoculation

Prior to flow cell inoculation the flow of media was paused, the tubing between the bubble traps and the flow cells was clamped and the waste bottles placed higher than the flow cell to prevent air being drawn into the system and media washing back through the tubing. To allow for a downstream flow as the flow cells were inoculated, the pump tubing was also released from the pump cassettes. In addition, tubing at the site of inoculation was sterilised using 80% (v/v) ethanol. Using a 1 mL 27-gauge syringe, 800 µL of bacterial suspension was drawn up and 400 µL expelled from the syringe to remove any bubbles. The syringe was then inserted into the tubing and manipulated into the inlet port of the flow cell where 300 µL of the bacterial suspension was slowly injected into the chamber. The needle puncture site was then sealed using silicon glue. For each flow cell, individual chambers were inoculated with one of the three biological replicates for every isolate selected for testing. After all three chambers were inoculated the flow cell was inverted and incubated for two hours. After incubation, the pump tubing was reattached, the metal clamps released and the flow cell returned to an upright position. The waste bottles were lowered to below the height of the system and the pump was then resumed at 3.0 mL per hour per channel (50 µL/minute) .The flow cell system was incubated at 30 °C for 72-hours. Each day the tubing upstream of the flow cell was thoroughly checked for bacterial overgrowth and bubble formation in the chambers.

5.3.5.7 Flow Cell Effluent Collection and Analysis

After the 72-hour incubation period, an aliquot of the media was collected from downstream of the peristaltic pump to assess colonial morphology and total colony forming unit (CFU) counts of dispersed cells arising from each flow cell. Approximately 1 mL of waste media was aseptically collected and serially diluted (Neat, 10^{-2} to 10^{-8}) in 0.9% NaCl (Sodium Chloride, normal saline). Ten microlitres of each diluted sample was then spread onto LB agar, incubated aerobically at 37 °C for 72-hours with the mean CFUs/mL for each organism determined from across the CFU counts of respective biological replicates (Kirov *et al.* 2007). The combined total number of different

colonial morphotypes arising from the effluent of each isolate was also recorded, with a representative of each stored in 15% glycerol at -80 °C for future analysis.

5.3.5.8 Flow Cell Staining

Following effluent collection the pumps were stopped, the media flow halted and tubes clamped as per the methodology described in the inoculation of flow cells (Section 5.3.5.6). A final volume of 300 µL of 10020 nM FilmTracer™ LIVE/DEAD® Biofilm Viability Kit stain (Invitrogen™, Thermo Fisher Scientific Inc, Newstead, Australia), was the injected into each chamber as per injection method described previously. This stain composed of the nucleic acid stains SYTO®9 and propidium iodine, to facilitate the visualisation of live intact cells and dead cell, respectively. Each flow cell was protected from light and incubated for 20 minutes followed by a 10-minute media wash at 3.0 mL per hour per channel (50 µL/minute).

5.3.5.9 Microscopy

Following staining the bacterial structure was imaged using a Zeiss 780 NLO confocal laser scanning microscope (CLSM; Carl Zeiss Microscopy, Oberkochen, Germany). For each flow cell four Z-stacks (0.71 µm intervals) were imaged at a distance of 1 cm from the inlet valve using a Plan-Apochromat 63x/1.4 oil DIC M27 objective lens. The SYTO9 stain was used to visualise the live cells via the excitation and emission wavelengths of 488 nm and 523 nm, respectively. Propidium iodine using excitation and emission wavelengths of 561 nm and 620 nm, respectively, was used to visualise the dead cells.

5.3.5.10 Flow Cell Data Analysis

Analysis of the flow cell CLSM results was undertaken by both qualitative and quantitative methods. Qualitative assessments were undertaken by categorically defining the biofilm characteristics and structures of each isolate tested (Figure 5.2). Cellular adhesion to the substratum (glass coverslip) was described as either a patchy uneven distribution of cells forming an irregular monolayer (Heterogeneous; Figure 5.2A), or a uniform growth of cells, forming an even monolayer (Homogeneous; Figure 5.2B). Two previously defined quantitative descriptions were used to categorise the three dimensional cellular structures identified within the flow cell chambers (Petrova *et al.* 2012). Microcolonies (Figure 5.2C, D, G and H) were defined as larger cellular formations measuring greater than 50 µm in diameter and or greater than 30 µm in height, whereas small cellular formations measuring less than 50 µm in diameter and less than 30 µm in height were categorised as cell clusters (Figure 5.22E and F).

Quantification of the CLSM z-stack results was achieved using Comstat version 2.1 software (Heydorn Micro 2000, Vorregaard tech Uni of Denmark 2008, www.comstat.dk). The following parameters were calculated: biomass ($\mu m^3/\mu m^2$), maximum thickness (µm) and surface area (μm^2).

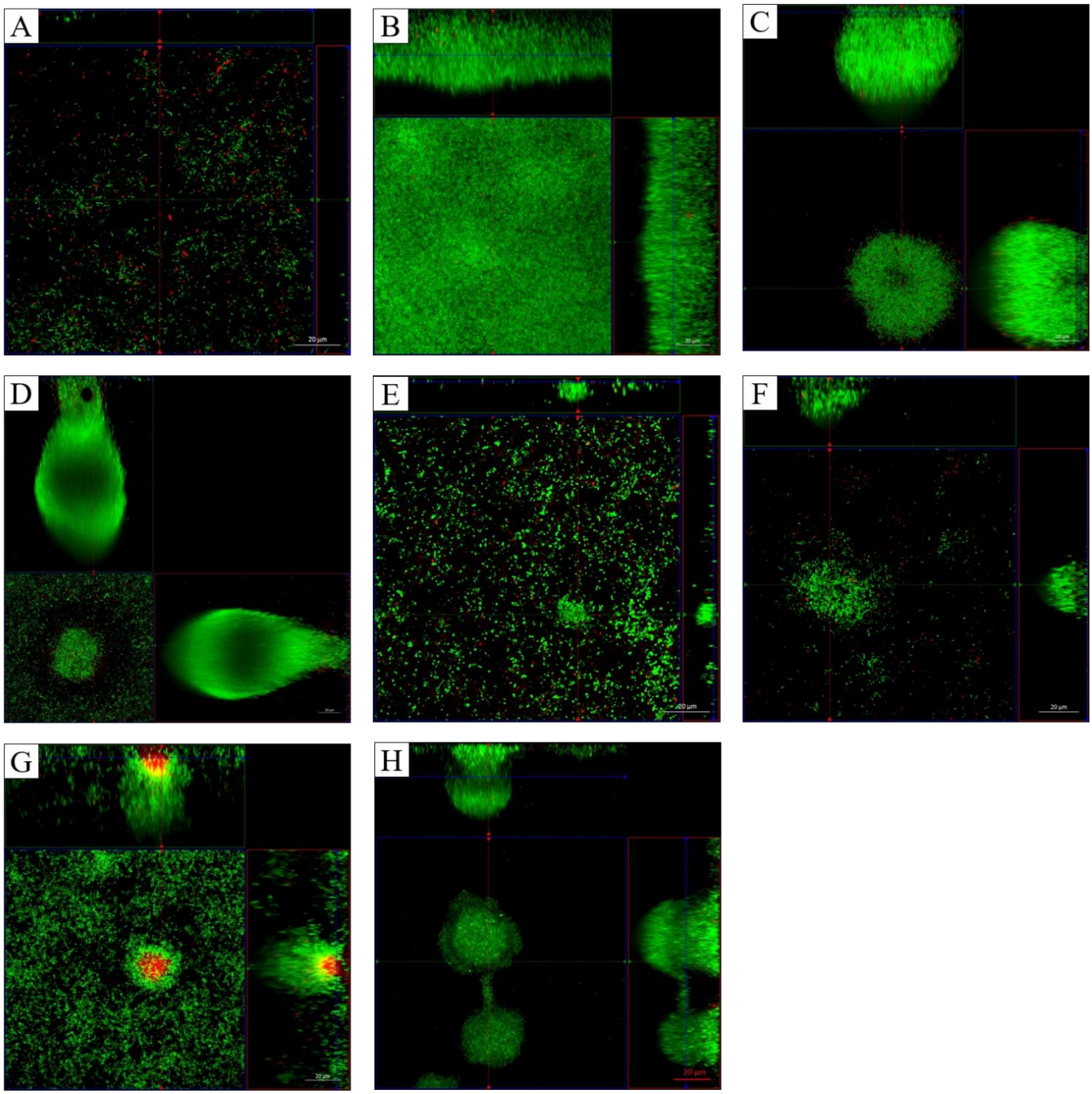


Figure 5.2 Examples of various flow cell biofilm architectural features.

A) A heterogeneous monolayer demonstrating poor attachment to the substratum; B) A homogeneous monolayer demonstrating uniform cellular attachment to the substratum; C & D) Microcolonies (≥ 50 µm diameter ± ≥ 30 µm height); E & F) Cell clusters (< 50 µm diameter and < 30 µm height); G) A microcolony with dead cells (red) at the centre; H) Microcolonies joined by a 'bacterial bridge' (Scale 20 µm).

5.3.6 Statistical Analysis

Categorical data, niche (environmental or CF shared strain, AUST-02) versus phenotype (motility and adherence) in different atmospheric conditions (aerobic, microaerophilic, anaerobic) were examined using Chi-square with Yates continuity correction to confirm the same association as previous reported in Chapter 4. Statistical analysis was performed using SPSS version 22, with a P value ≤ 0.05 indicating statistical significance.

To account for replicates in both flow cell and adhesion assays, individual results were combined and compared as follows.

Flow Cell Assay Analysis: For each experiment, one flow cell, consisting of three chambers (Chamber 1[C-1], Chamber 2[C-2] and Chamber 3[C-3]) were each inoculated with biological replicates of an individual isolate, therefore triplicates of each isolates were prepared for biofilm analysis. Four z-stacks were captured from each chamber; therefore a total of 12 z-stacks were recorded. Quantification of z-stacks was undertaken using the computer program Comstat. All z-stack results generated from Comstat for the environmental isolates in C-1 were combined, as too were the results from isolates tested in C-2 and again for the isolates tested in C-3. Z-stack results from the AUST-02 strains were also combined according to chambers. Results from the i) environmental isolates grown in C-1 were compared to the AUST-02 strains grown in C-1, ii) environmental isolates grown in C-2 were compared to the AUST-02 strains grown in C-2, and iii) environmental isolates grown in C-3 were compared to the AUST-02 strains grown in C-3. Mixed effects model, Pearson correlation coefficient and density estimate curves all used these data comparisons to be calculated.

Microtitre Plate-Based Assay Analysis: Three technical replicates (Well 1[W-1], Well 2 [W-2] and Well 3[W-3]) of each isolate were assessed at two different time points (biological replicates, Replicate 1[R-1] and Replicate 2[R-2]). To assess results across replicates (W-1, W-2 and W-3) and time points (R-1 and R-2), all results obtained in W-1 at both time points (R-1 and R-2) were combined. This was repeated for results obtained from W-2 and W-3 at time points, R-1 and R-2. Therefore, the results from the: i) environmental isolates grown in W-1 (R-1 and R-2 combined) were compared to the AUST-02 strain grown in W-1 (R-1 and R-2 combined), ii) environmental isolates grown in W-2 (R-1 and R-2 combined) were compared to the AUST-02 strain grown in W-2 (R-1 and R-2 combined), and iii) environmental isolates grown in W-3 (R-1 and R-2 combined) were compared to the AUST-02 strain grown in W-3 (R-1 and R-2 combined). Mixed effects model used these data comparisons to be calculated.

Mixed effects models with replicate nested within specimen identification including fixed effects, were performed to compare niche effect with interaction with time. Density estimate curves, using Gaussian kernel, were generated to compare strains.

Least squared estimates and 95% confidence intervals included fixed effects with their interactions. For variables that showed considerable skewness, the data was transformed with log (1+x) and the linear modelling was performed on the log scale. Comparisons were not adjusted for multiple testing. Statistical analyses were performed in R (R Core Team, 2017) with the *lsmeans*, lme4 and the MAd packages.

5.4 Results

5.4.1 Colonial morphology and motility

Subset analyses of colonial pigmentation, mucoidy and swim, swarm and twitching motilities for the 14 (environmental [n = 6] and AUST-02 [n = 8]) isolates revealed similar results to those obtained in Chapter 4. All six of the environmental isolates displayed a non-mucoid colonial morphotype, with four also showing pyocyanin-related blue-green pigmentation when grown on LB agar. Only one AUST-02 isolate displayed a mucoid colonial morphotype and five produced pigmentation (one showed pyocyanin-related blue-green pigmentation and four showed pyomelanin-related brown pigmentation) on LB agar (Table 5.1). When swimming, swarming and twitching motility was assessed, the environmental isolates displayed substantial phenotypic heterogeneity, whereas the motility attributes of the AUST-02 strain isolates were much more tightly clustered (Figure 5.3A-I). Nevertheless, with the exception of anaerobic twitching motility, isolates collected from the environment showed significantly enhanced motility across the range of atmospheric conditions as compared to AUST-02 (Figure 5.3A-I).

5.4.2 Microtitre Plate-based Assay Analysis

Figure 5.3J-L shows that compared to the AUST-02 strain isolates, the six environmentally-derived isolates displayed an enhanced capacity to adhere to a polystyrene surface as demonstrated by the static microtitre plate-based assay. Consistent with the findings of Chapter 4, significant differences in bacterial adherence between the two groups were noted in all aerobic ($P = 0.005$), microaerophilic ($P = 0.005$) and anaerobic ($P = 0.005$) conditions. Likewise, when analysed in the mixed effects model comparing ecological niche by replicate analysis, the environmental isolates also showed an increased propensity for cellular adhesion across all atmospheric conditions (Table 5.2; $P \leq 0.005$).

Repeat of the microaerophilic microtitre plate-based assay using minimal M63 media demonstrated similar results to that of LB broth with the environmental isolates showing increased adherence in the model (Figure 5.3M; $P = 0.031$). However, although all the environmental isolates retained the ability to adhere to the polystyrene surface, there was an overall reduction in the degree of binding noted. In contrast, adherence of the AUST-02 strains was not reduced in minimal media, with two of the isolates demonstrating improved adherence to the microtitre plate as compared to microaerophilic growth in LB. Altogether, these data suggest that nutrient restriction not only decreases bacterial generation, but may also impact on cellular adherence to abiotic surfaces.

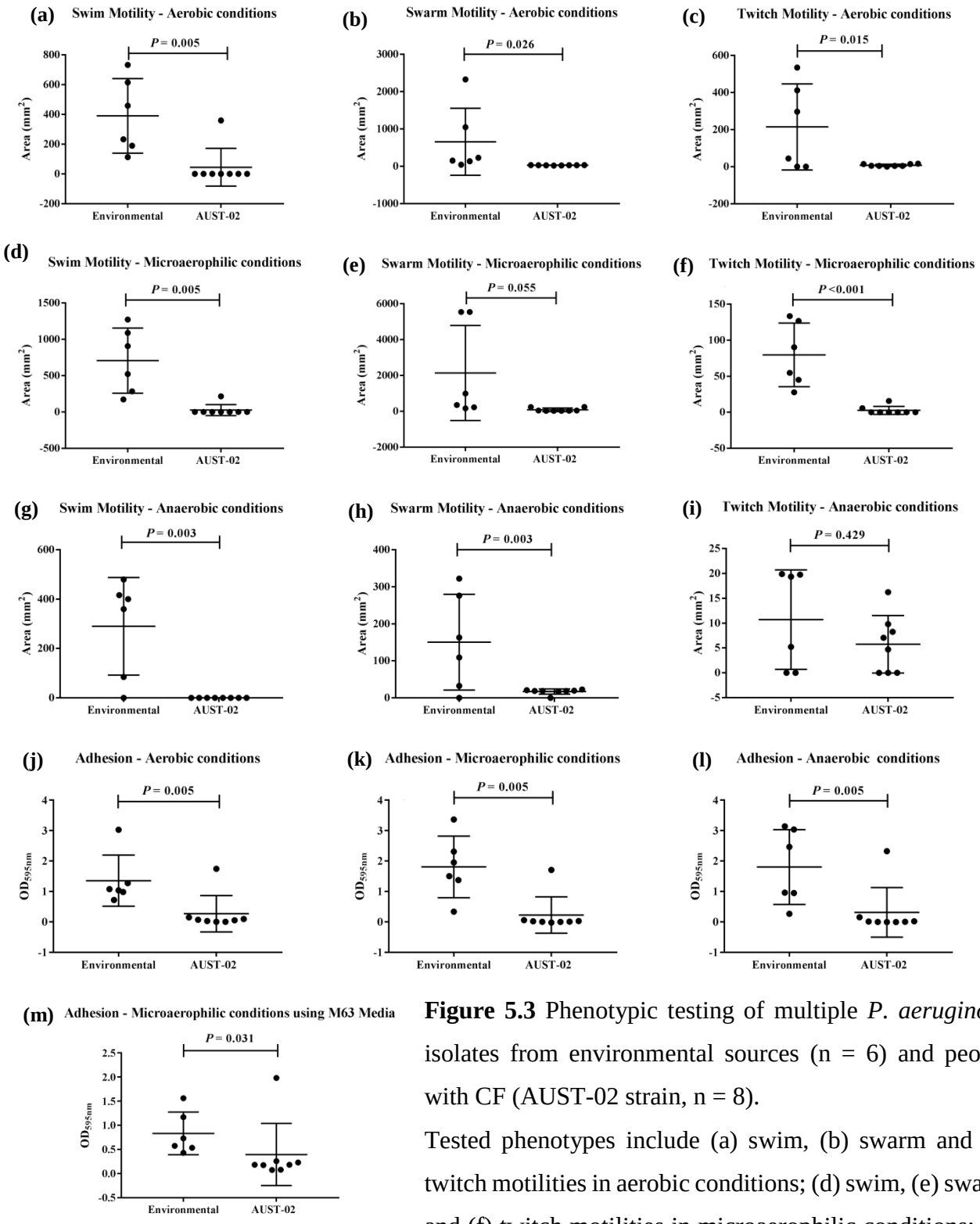


Figure 5.3 Phenotypic testing of multiple *P. aeruginosa* isolates from environmental sources (n = 6) and people with CF (AUST-02 strain, n = 8).

Tested phenotypes include (a) swim, (b) swarm and (c) twitch motilities in aerobic conditions; (d) swim, (e) swarm and (f) twitch motilities in microaerophilic conditions; (g) swim, (h) swarm and (i) twitch motilities in anaerobic conditions; adhesion results from the microtitre plate-based assay using LB media in (j) aerobic, (k) microaerophilic and (l) anaerobic conditions and (m) adhesion results from the microtitre plate-based assay using M63 media in microaerophilic conditions. The average motility result tested in triplicate and six replicates from the microtitre assay are presented, with error bars representing the mean and standard deviation.

Table 5.2 Mixed effects model comparing optical density measurements from the plate-based adhesion assays for niche by replicate analysis of environmental isolates (n = 6) and AUST-02 strains (n = 8) following 24-hours incubation.

Atmospheric condition	AUST-02 Strains (n = 8)		Environmental Isolates (n = 6)		*P*-value
	Replicate 1	Replicate 2	Replicate 1	Replicate 2	
	lsmeans (95% CI)	*lsmeans* (95% CI)	*lsmeans* (95% CI)	*lsmeans* (95% CI)	
Aerobic	0.33 (-0.22 - 0.88)	0.20 (-0.35 - 0.75)	1.33 (0.70 - 1.97)	1.37 (0.74 - 2.01)	0.005
Microaerophilic	0.17 (-0.44 - 0.78)	0.28 (-0.33 - 0.89)	1.98 (1.27 - 2.68)	1.62 (0.92 - 2.33)	< 0.001
Anaerobic	0.33 (-0.45 - 1.11)	0.29 (-0.49 - 1.07)	1.95 (1.05 - 2.85)	1.66 (0.76 - 2.56)	0.006

5.4.3 Bacterial growth curve analysis

From the growth curve analysis and calculation of the generation time, there appeared to be a fitness cost associated with the AUST-02 strains. Compared to the control strain, PAO1 (mean doubling time, 29 minutes) and the environmental isolates (mean doubling time, 43 minutes), the AUST-02 strains (mean doubling time, 92 minutes) was slower to grow (Figure 5.4).

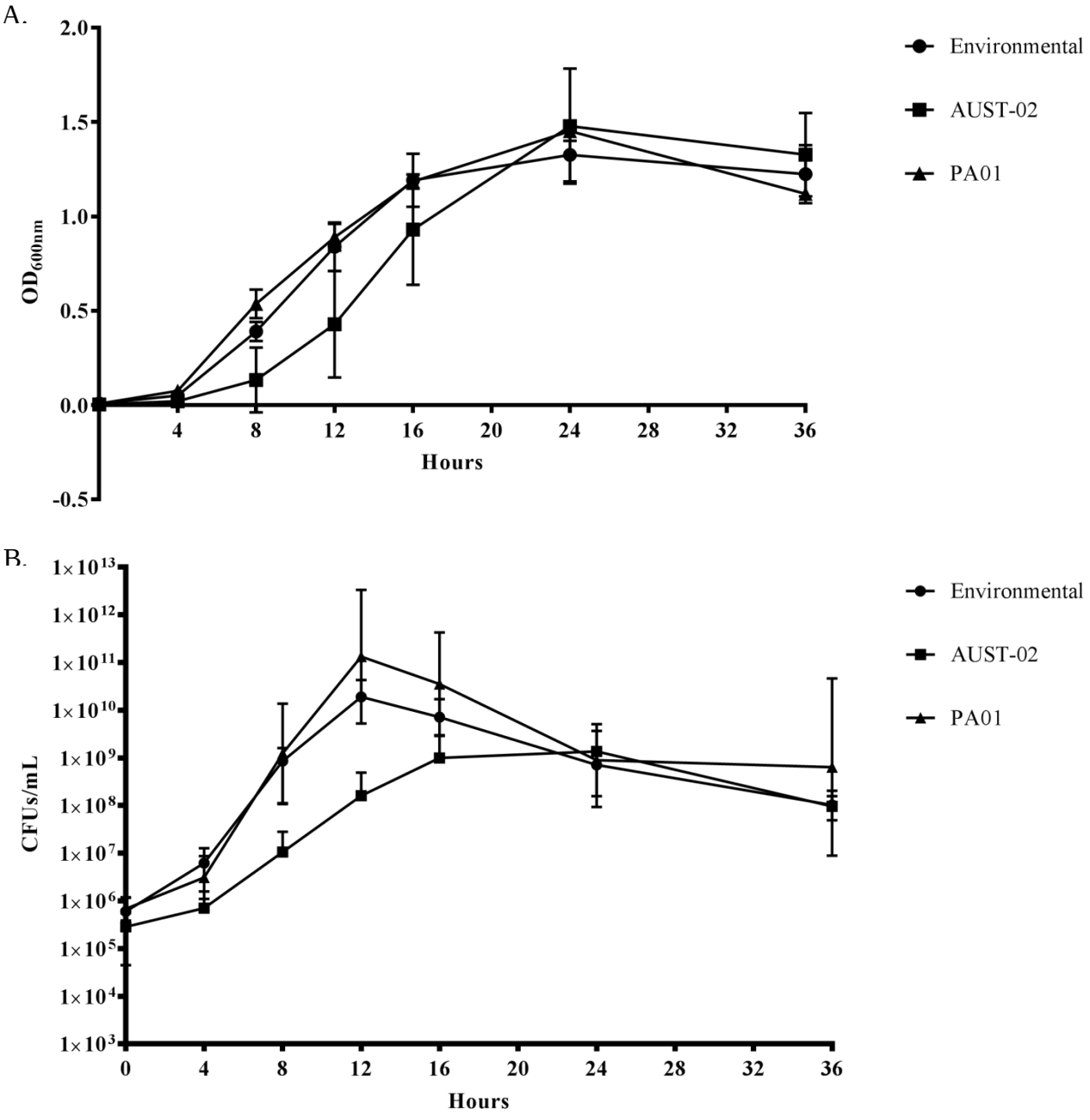


Figure 5.4 Growth curve results displaying the A) bacterial density (OD_{600nm}) and B) CFUs/mL of the 18 isolates to undergo analysis in the flow cell apparatus (environmental n = 6, AUST-02, n = 12) and control strain PAO1 (biological replicates, n = 4).

5.4.4 Qualitative Flow Cell Analysis

Results of the qualitative flow cell analyses are presented in Table 5.3. A total of 76 CLSM z-stacks from the six environmental isolates were reviewed with cellular structures identified in 57% of images, with the majority of containing only one cellular structure (70%) per image. Collectively, a total of 63 environmental isolate biofilm structures were reported, 26 (41%) of which were defined as microcolonies. There was no difference in the number of environmental isolates which demonstrated either homogeneous adherence to the coverslip or poor adhesion and a heterogeneous monolayer. The proportion of microcolonies and cell clusters formed by the environmental isolates was similar to the reference strain, PAO1.

In contrast to microtitre plate-based model, all eight of the AUST-02 isolates were capable of adhering to the glass substratum used the flow cell apparatus. Similar to the environmental isolates, 47 (49%) of the 97 AUST-02 CLSM z-stacks reviewed showed a homogeneous biofilm monolayer. Cellular structures (n = 129), including both cell clusters and microcolonies were identified in 62% of all the AUST-02 images captured. Thirty-five (27%) and 94 (73%) were categorised as cell clusters and microcolonies, respectively.

When comparing the environmental and AUST-02 strains it was revealed that both groups shared many similar structural characteristics when grown in the flow cell chambers. There was no difference in the proportion of images containing cellular structures ($P = 0.585$), nor was there any disparity in homogenous monolayer development at the substratum ($P = 0.364$). However, when all the cellular structures, in each z-stack were categorised into either small cell clusters or the larger microcolony structures, the AUST-02 strains produced significantly more microcolonies than the environmental isolates ($P < 0.001$). Furthermore, the AUST-02 isolates produced substantially more cellular structures per z-stack compared to the environmental isolates. On average there were 2.2 cellular structures identified per z-stack from the AUST-02 strain compared to 1.5 reported from the environmental isolates. While the height of the cellular structures was greater for the AUST-02 strains ($P = 0.010$), the environmental isolates produced microcolonies with larger diameters ($P = 0.021$). Representative images of the AUST-02 and environmental isolates can be found in Appendix 5.1.

Table 5.3 Descriptive features of biofilm characteristics.

Parameter	PAO1	Environmental isolates	AUST-02 strains	*P*-value [a]
Number of isolates analysed	1	6	8	
Total number of z-stacks reviewed	13	76	97	
Individual z-stack features				
Homogeneous monolayer; z-stacks, n (%)	6 (46.2)	43 (56.6)	47 (48.5)	0.364
Cellular structures *; z-stacks, n (%)	13 (100.0)	43 (56.6)	60 (61.9)	0.585
Number of all cellular structures per z-stack:				
1	3 (23.1)	30 (69.8)	34 (56.7)	
2	4 (30.8)	7 (16.3)	9 (15.0)	
3	3 (23.1)	5 (11.6)	6 (10.0)	
4	2 (15.4)	1 (2.3)	5 (8.3)	
5	1 (7.7)	0 (0.0)	2 (3.3)	
6	0 (0.0)	0 (0.0)	1 (1.7)	
7	0 (0.0)	0 (0.0)	2 (3.3)	
8	0 (0.0)	0 (0.0)	0 (0.0)	
9	0 (0.0)	0 (0.0)	1 (1.7)	
Total number of microcolonies detected, n (%)	18 (54.5)	26 (41.3)	94 (72.9)	< 0.001
Total number of cell clusters detected, n (%)	15 (45.5)	37 (58.7)	35 (27.9)	< 0.001
Size of all cellular structures				
Diameter µm, mean ± SD (range)	38.2 ± 16.0 (11.6 - 64.8)	38.6 ± 17.8 (13.7 - 89.6)	39.0 ± 16.1 (11.9 - 84.6)	0.880
Height µm, mean ± SD (range)	34.7 ± 22.2 (8.6 - 84.3)	33.3 ± 26.9 (7.9 - 162.1)	41.9 ± 18.3 (6.4 - 92.2)	0.010
Size of microcolonies				
Diameter µm, mean ± SD (range)	47.2 ± 11.6 (28.9 - 64.8)	52.2 ± 18.9 (21.1 - 89.6)	44.3 ± 15.1 (18.9 - 84.6)	0.021
Height µm, mean ± SD (range)	49.7 ± 19.6 (11.4 - 84.3)	54.5 ± 30.9 (21.4 - 162.1)	50.1 ± 14.0 (30.7 - 92.2)	0.294

[a] A two-way unpaired t-test was performed between environmental and AUST-02 isolates only.

* Cell clusters and microcolonies combined.

5.4.5 Quantitative Flow Cell Analysis

Quantitative assessment of the CLSM images using Comstat analysis software demonstrated that compared to the environmental isolates, the AUST-02 isolates displayed enhanced biofilm formation. Mixed effects models, presenting least square means determined that the AUST-02 strains formed thicker biofilms ($P < 0.001$), with increased biomass ($P = 0.012$) and greater surface area coverage ($P = 0.002$) than the environmental isolates, when assessing the live cells comprising the biofilm only (Table 5.4). These data were further supported by the results from the Pearson Correlation Co-efficient analyses which demonstrated that the Comstat live cell parameters displayed a strong correlation with each of the other parameters (Table 5.5). Therefore, this data demonstrates that biofilms comprising of greater biomass will also be thicker and cover more surface area.

To complement the Comstat-based analyses we also used density curves estimates to visualise and compare the log biomass, maximum thickness and surface area of the environmental and AUST-02 isolate groups across the three flow cell chambers (Figure 5.5). It was observed that the three curves produced by the environmental isolates, representing combined results generated from chambers 1, 2 and 3, clustered tightly together for biomass, maximum thickness and surface area coverage. Likewise, the AUST-02 isolate curves showed similar clustering, but these were distinct from the environmental isolate curve clusters. The AUST-02 curves also showed increased percent density as compared to the environmental isolate curves.

Altogether, the quantitative analyses of biofilm growth and architecture within the flow cell apparatus indicated that the AUST-02 isolates produced larger biofilms structures as compared to the environmental isolates (Table 5.3). Coupled with the qualitative assessments these data confirm that the AUST-02 strain is capable of forming robust biofilm structures and can adhere to a glass substratum.

5.4.6 Reproducibility of *in vitro* biofilm methodologies

As previously described in Chapter 4, when using Luria Broth as the growth medium, a high degree of reproducibility was determined for the microtitre plate based assay. Briefly, intra class correlations (ICC) determined that agreement measures for this assay were excellent (ICC: aerobic 0.972 [95% CI: 0.966 – 0.977] $P < 0.001$, microaerophilic 0.986 [95% CI: 0.984 – 0.989] $P < 0.001$, anaerobic 0.986 [95% CI: 0.983 – 0.989] $P < 0.001$). As this methodology was determined to be highly reproducible, this analysis was not repeated when testing was conducted under microaerophilic conditions using minimal growth medium.

Density estimate curves were used to assess the reproducibility of the flow cell results. As demonstrated in Figure 5.5, the three curves produced by the environmental isolates, representing combined results generated from chambers 1, 2 and 3, clustered tightly together for biomass, maximum thickness and surface area coverage. Therefore, there was a high degree of reproducibility across chambers and between experiments. Results from the AUST-02 strains also demonstrated high levels of reproducibility.

Table 5.4 Mixed effects model comparing quantitative live cell parameters generated from environmental (n = 6) and AUST-02 strains (n = 8) following 72-hours of growth within the flow cell model.

	Live Cells stained with SYTO9		
Biofilm Parameter	AUST-02 Isolates (n = 8) *lsmeans* (95% CI)	Environmental Isolates (n = 6) *lsmeans* (95% CI)	*P*-value
Biomass ($\mu m^3/\mu m^2$)	1.66 (1.15 – 2.17)	1.21 (0.64 – 1.78)	0.012
Maximum Thickness (μm)	3.91 (3.66 – 4.16)	3.48 (3.21 – 3.76)	< 0.001
Surface Area (μm^2)	12.55 (12.13 – 12.98)	12.08 (11.61 – 12.55)	0.002

Table 5.5 Results of Pearson Correlation Coefficient comparing environmental and AUST-02 isolate (n = 14) biofilm measurements as determined by Comstat for the live cells in combined z-stacks from CLSM analysis.

Biofilm Measurements	Live cells - SYTO9	
	r	*P*-value
Biomass vs. Maximum Thickness	0.65	< 0.001
Biomass vs. Surface Area	0.70	< 0.001
Maximum Thickness vs. Surface Area	0.44	< 0.001

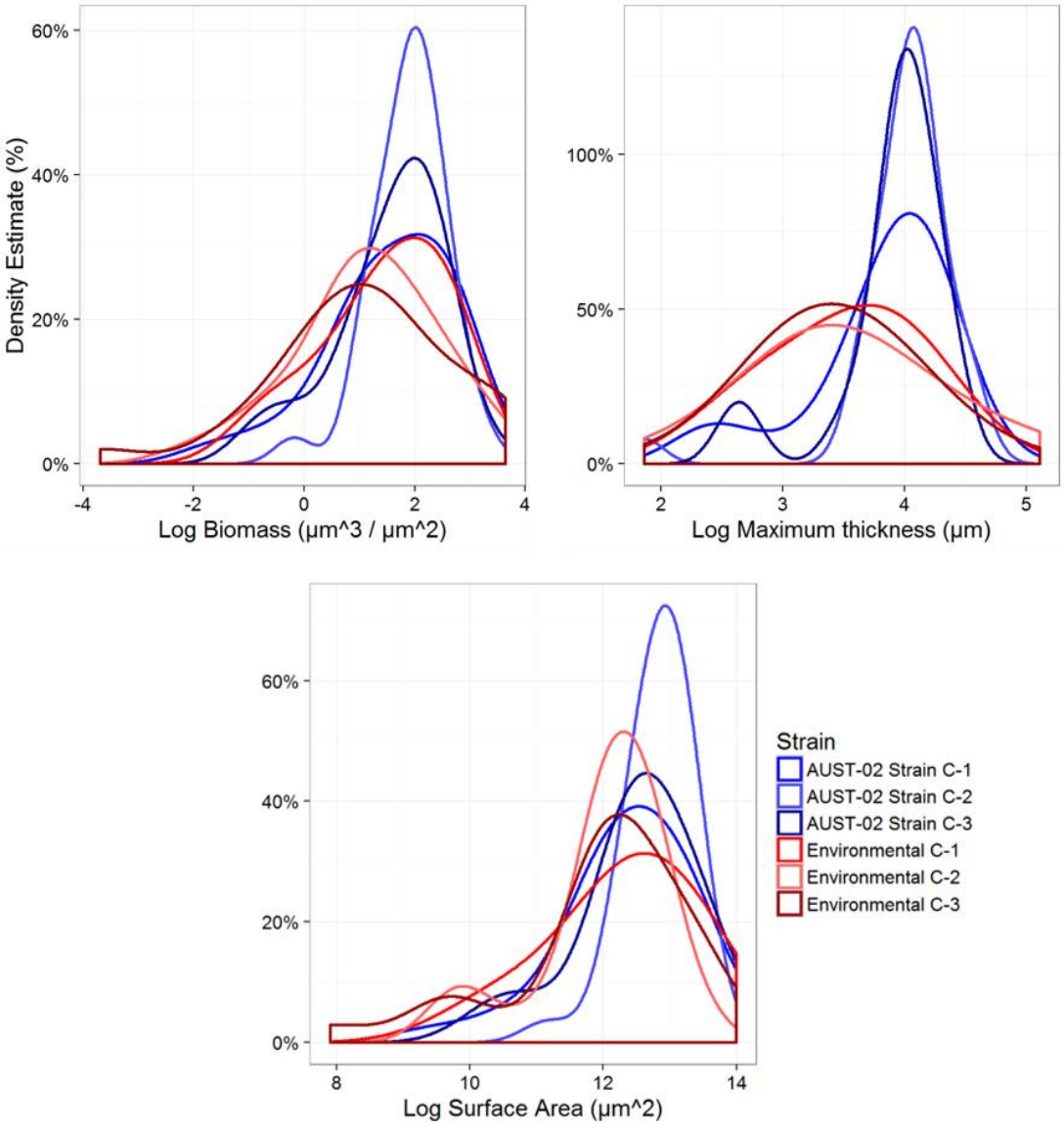


Figure 5.5 Density estimate curves (i.e. biomass, maximum thickness and surface area of the live biofilm cells) showing clustering of the two isolate groups and the reproducibility of the flow cell methodology.

All results obtained from chamber 1 (C-1) across each independent experiment were combined and compared with all results obtained from chamber 2 (C-2) and chamber 3 (C-3) for environmental isolates and AUST-02 strains.

5.4.7 Effluent Isolate Analysis

Each individual flow cell experiment generated between one and four morphologically distinct presumptive *P. aeruginosa* isolates from the effluent collected at the conclusion of each experiment. All isolates retrieved from the effluent were non-mucoid. Pigmentation was produced by 64% of

isolates grown in the effluent of the environmental isolates, which reflected the morphotypes of the 'parent' isolates. Whereas, there was a significant increase in the number of dispersed cells from the AUST-02 strains displaying pigmentation (92%) compared to original 'parent' strain (50%) on which the experiment was conducted (Fisher's Exact Test $P = 0.009$).

The overall number of CFUs/ml enumerated from the effluent samples was significantly greater for the AUST-02 isolate group (Students T-test $P = 0.008$) (Table 5.6). As previously shown the AUST-02 strains developed more robust flow cell biofilms compared to the environmental strains. Therefore the analysis was repeated to account for any influence which biofilm biomass or surface area may have on bacterial shearing and/or cell dispersal. Following adjustment, the difference in CFUs/ml obtained from the bacterial effluent was no longer significant between the two isolate groups (biomass adjusted $P = 0.092$; surface area coverage adjusted $P = 0.121$), indicating the size of the biofilm is a contributing factor to bacterial dispersal.

Table 5.6 Number of morphologically distinct isolates and average CFU/ml collected from the long-term flow cell following 72-hours incubation at 37 °C for the isolates included in the environmental and AUST-02 comparison analysis (n = 14).

Isolate Number	Source	Sequence Type	Average CFUs/ml per isolate	Maximum number of morphotypes detected across 3 chambers
1	Environment	155	8.9×10^7	3
2	Environment	179	1.1×10^7	2
3	Environment	253	9.9×10^7	4
4	Environment	266	1.2×10^7	2
5	Environment	381	1.3×10^8	1
6	Environment	471	3.1×10^7	4
	Mean ± SD		**$6.2 \times 10^7 \pm 5.1 \times 10^7$**	**6.5 ± 2.9**
7	CF (Adult)	775	7.2×10^7	3
8	CF (Adult)	775	7.3×10^8	3
9	CF (Adult)	775	1.2×10^8	4
10	CF (Adult)	775	1.0×10^8	4
11	CF (Paediatric)	775	5.5×10^8	1
12	CF (Paediatric)	775	2.9×10^8	2
14	CF (Adult)	775	1.3×10^8	2
18	CF (Adult)	775	3.9×10^8	2
	Mean ± SD		**$3.0 \times 10^8 \pm 2.4 \times 10^8$**	**6.3 ± 2.3**
	PAO1		3.3×10^8	3

5.4.8 Longitudinally collected AUST-02 strains

Patient 1, a young child time at time of *P. aeruginosa* acquisition, was infected for 6 years prior to participation in this study. All isolates collected over the eleven year period retained a non-mucoid morphotype, however overtime these isolates became non-pigmented. Other than limited variations in swimming motility reported, these isolates were non-motile and non-adherent. Patient 2 was infected with *P. aeruginosa* for six years prior to sample collection during 2002. There was no change noted over time regarding morphology and similar to other AUST-02 strains, these isolates were predominantly non-motile and non-adherent (Table 5.7).

As demonstrated in Figure 5.6, despite the appearance of a downward trend, no change was noted for any biofilm parameter as assessed using Comstat software in either patient overtime (Biomass P = 0.089, maximum thickness P = 0.461, surface area P = 0.164). Furthermore, similar longitudinal trajectories were reported when comparing biofilm formation from Patient 1 with Patient 2 (Biomass P = 0.834, maximum thickness P = 0.508, surface area P = 0.869) (Table 5.8).

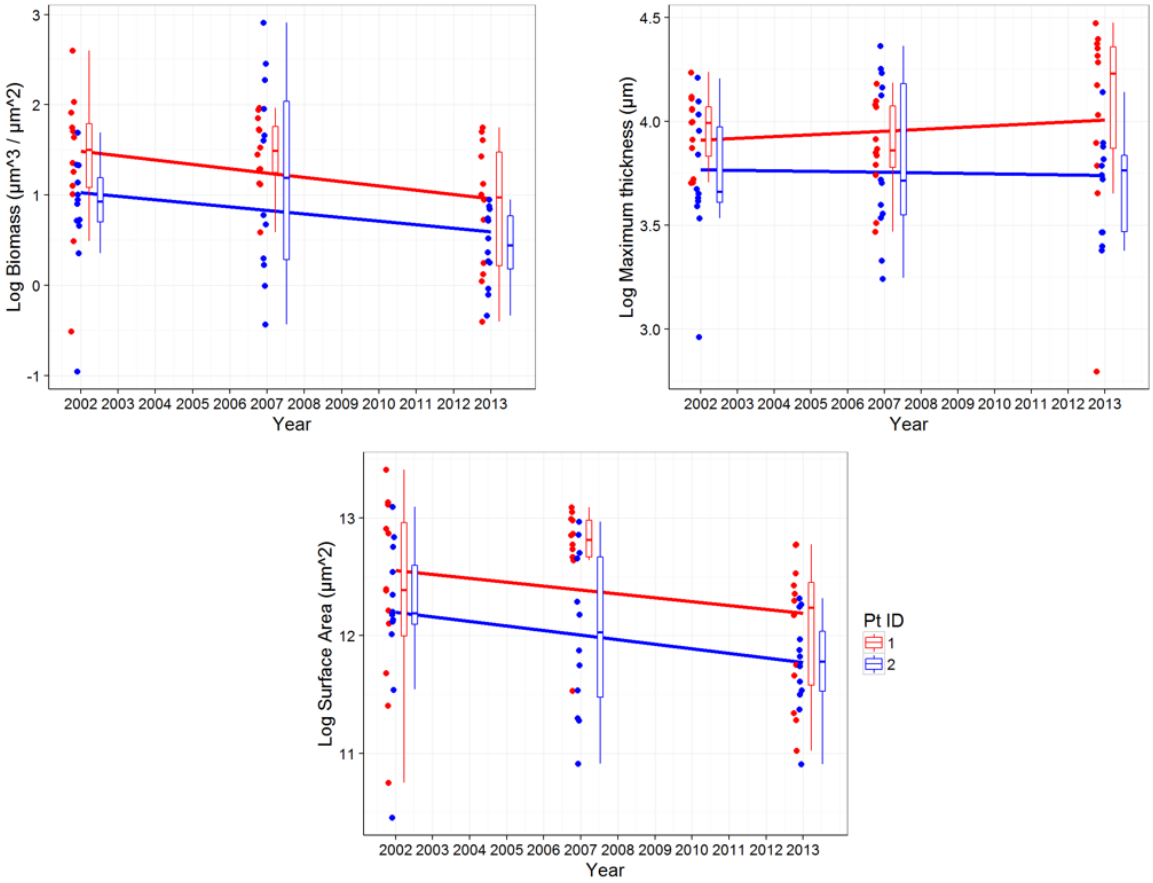


Figure 5.6 The change over time and comparison of longitudinal trajectories of biofilm forming characteristics obtained from Patient 1 (Pt ID 1) and Patient 2 (Pt ID 2), chronically infected with AUST-02, collected in the years 2002, 2007 and 2013.

Table 5.7 Results from the phenotypic and microtitre plate-based assays for the longitudinally collected isolated isolates from two patients chronically infected with AUST-02 (n = 6).

Isolate Number		CFTR	Year of collection	Age at collection	Swim			Swarm			Twitch			LB Media @ 24-hours			M63 under MA conditions at 24-hours
					O_2	MA	AnO_2	O_2	MA	AnO_2	O_2	MA	AnO_2	O_2	MA	AnO_2	
13			2002	16	-	+	-	-	-	-	-	-	-	-	-	-	-
14	Patient 1	p.F508del/N130K	2007	21	-	-	-	-	-	-	-	-	-	-	-	-	-
15			2013	27	-	-	+	-	-	-	-	-	-	-	-	-	-
16			2002	14	-	-	-	-	-	-	-	-	-	-	-	-	+
17	Patient 2	p.F508del/p.F508del	2007	19	-	-	-	+	-	-	-	-	-	-	-	-	-
18			2013	25	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 5.8 Comparison of biofilm production between two patients chronically infected with the AUST-02 strain during the years 2002, 2007 and 2013.

	Live Cells stained with SYTO9	
Biofilm Parameters	Change over time	Longitudinal Trajectory (Patient 1 vs Patient 2 over time)
	P-value	*P*-value
Biomass ($\mu m^3/\mu m^2$)	0.089	0.834
Maximum Thickness (μm)	0.461	0.508
Surface Area (μm^2)	0.164	0.869

5.5 Discussion

Using isolates obtained from the environment and the airways of people with CF this study determined that not only do these isolates differ phenotypically, but assays designed to infer similar mechanistic traits can report discordant results. More specifically, this study demonstrated that bacterial motility and adhesion are not unequivocally linked with an organism's ability to form robust long term biofilms. These results show for the first time that although the CF shared strain, AUST-02, showed subdued motility and poor adherence to an abiotic surface, compared to environmental isolates, it exhibited an enhanced capacity to form biofilms within a flow cell model. Furthermore, under these experimental conditions there did not appear to be any differences noted in the biofilm forming capacity of isolates collected longitudinally from patients with chronic AUST-02 infection. Overall, these data not only reveal novel insights into the infection biology of the prevalent Australian shared strain, AUST-02, but it also draws attention to the fact that some *in vitro* biofilm models may not be suitable for assessing the 'global' biofilm forming capacity of clinical isolates.

Bacteria have two predominant modes of growth – as a free-living (or planktonic) single cells, or within sessile structured communities referred to as biofilms (Costerton *et al.* 1995). Biofilms adhere to biotic or abiotic surfaces, and more recently cellular aggregates consisting of bacteria adhering to each other have been described (Alhede *et al.* 2014; Alhede *et al.* 2011; Bjarnsholt 2013; Costerton *et al.* 1995; Rybtke *et al.* 2015). Biofilm growth provides protection to the bacterial cells from exposure to antimicrobial substances and fluctuations in environmental conditions. During the transition from a planktonic to a biofilm lifestyle the bacteria undergo many mechanistic changes (Costerton *et al.* 1995; Hogardt and Heesemann 2010; Stoodley *et al.* 2002; Winstanley *et al.* 2016). Two well described phenotypic characteristics associated with biofilm development are bacterial motility and adhesion capability. Overwhelmingly, the data currently presented in the scientific literature describes the ability to adhere and the loss of motility to be correlated with biofilm formation. However, not all studies demonstrate such a clear link between these phenotypic characteristics and the enhanced ability to develop biofilms.

To more accurately assess biofilm development of the selected bacterial strains, all isolates were grown within a continuous flow cell apparatus. Eight AUST-02 strains possessing the same MLST profile were compared with six environmental isolates belonging to different genetic backgrounds. Despite differences in origin and genetic composition of these strains, all isolates shared a similar capacity for adherence and the generation of a homogeneous cellular monolayer within this flow cell model. However, during closer assessment of the bacterial structures comprising the biofilm, it was revealed that AUST-02 strains produced biofilm structures that were thicker, covered more surface

area and contained more biomass than those from environmental sources. Finally, the AUST-02 strains produced significantly greater numbers of microcolonies than the environmental isolates, though it should be noted that the size of these structures across the two groups were not dissimilar.

These data highlight the adaptability of *P. aeruginosa* and demonstrate that irrespective of the genetic background or origin of individual strains, all isolates possess the traits and mechanisms which enable them to form biofilms. It is difficult to compare or contrast these results with published data as this was the first study to assess biofilm formation of the AUST-02 strain in a continuous flow cell model. Furthermore, of the data currently available in the published scientific literature there is little concordance regarding methodologies and experimental conditions, rendering direct comparisons difficult. Nevertheless, several studies have assessed biofilm formation and motility among non-clinical and clinical strains, including those belonging to prevalent shared CF strains.

Due to the ease of use, high throughput and cost effectiveness the static biofilm model, in various forms, is most often used to infer adhesion and biofilm formation capabilities. To date, much of the current work available on this topic has focused on shared CF strains with high prevalence and associated with poorer clinical outcomes. These strains include isolates belonging to the Liverpool epidemic strain, prevalent throughout the UK and Canada, and the AUST-01 strain found within the Australian CF population. Despite the use of 96-well plates and similar experimental conditions, discordant results have been obtained for isolates belong to the LES. When compared to PAO1, two independent reports demonstrate reduced adherence (Cullen *et al.* 2015; Jeukens *et al.* 2014), whereas a further two studies observed enhanced adherence for the same strains (Carter *et al.* 2010; Kukavica-Ibrulj *et al.* 2008). Interestingly, while the study conducted by Kukavica-Ibrulj and colleagues reported no correlation with motility characteristics, similar to adhesion, the studies conducted by Cullen and colleagues and Jeukens and colleagues reported a reduction in motility compared to PAO1 (Cullen *et al.* 2015; Kukavica-Ibrulj *et al.* 2008).

Bacterial adhesion of the AUST-01 strain has also been assessed in various static biofilm models. Compared to PAO1, reduced adherence was observed when assessed in the microtitre plate, glass coverslip and borosilicate glass tube models (Cullen *et al.* 2015; O'May *et al.* 2006). In addition, these studies reported impaired motility compared to PAO1 which is similar to other work comparing other shared CF strains with those isolated from other clinical presentations (Cullen *et al.* 2015; Head and Yu 2004; Jeukens *et al.* 2014; O'May *et al.* 2006). In this study, the shared strain, AUST-02 displayed impaired motility and reduced adhesion under static conditions, when compared to both the PAO1 reference strain and a number of environmental isolates.

Given the complexity of assessing biofilm formation using the continuous flow cell model, fewer studies have been conducted. However, one study has demonstrated that LES can produce biofilms containing greater biomass when compared with PAO1 (Rojo-Molinero *et al.* 2016). Likewise, AUST-01 has been shown to produce thicker biofilms, covering a larger surface area as compared to both PAO1 and non-shared CF strains (Manos *et al.* 2008). Although CF strains, including AUST-01 were slower to attach and form a monolayer compared to PAO1 in a continuous flow model, Kirov and colleagues reported that by 24-hours microcolony development had occurred and at the cessation of the experiment, these strains had produced biofilms with comparable thickness to PAO1 consisting of well-defined structures (Kirov *et al.* 2007). Whilst similar levels of adhesion and monolayer attachment in the flow cell model were seen when AUST-02 was compared with both PAO1 and environmental isolates, this current study reported that biofilms produced by AUST-02 were of a greater biomass and consisted of more cellular structures. Collectively, both the current and earlier data do indicate that highly prevalent shared CF strains show an enhanced capacity for forming biofilms in continuous flow cell models.

A major finding of this study was the discordant results observed when assessing bacterial adhesion between the static microtitre plate-based assay and the continuous flow cell model. Despite using the same isolates and maintaining consistent experimental conditions throughout, the environmental isolates arising from various genetic backgrounds and sample sites demonstrated enhanced adhesion in both methods, whereas the AUST-02 strains only achieved a high level of adherence in the flow cell model. A number of earlier studies have also compared the adherence of both clinical and environmental isolates on different abiotic surfaces including glass and plastic. Overall, these data indicate that adhesion capabilities show uniformity across different substrates (Deligianni *et al.* 2010; Head and Yu 2004; Lee *et al.* 2005; O'May *et al.* 2006). Therefore, the between group differences in adhesion we observed here may indicate that the AUST-02 strain has a strain specific impairment in its capacity to adhere to polystyrene; however, several aspects of the experimental design also require careful consideration. Compared to the flow cell model which was conducted over an extended period with a continual supply of fresh media, the static microtitre plate-based model was performed over a shorter duration with a fixed supply of media and nutrients. As shown in the fitness experiments, under aerobic conditions, the AUST-02 generation time was substantially longer than that of the environmental isolates. Therefore, it is feasible that the length of the microtitre plated-based experiments may have been insufficient to enable adherence of the AUST-02 strains. Depletion of nutrients within the media may have also occurred which resulted in bacterial senescence or death prior to attachment. However, it was important note that when using the nutrient rich media, LB, the

same lack of adhesion was noted. As results from this experiment were only obtained following 24-hours of incubation, the precise impact of the growth rates and respiration under reduced oxygenation of the individual bacterial strains could not be concluded.

Few studies comment on the final growth stage of a biofilms life, the dispersal event, in which motile cells are actively expelled from the biofilm structure. In this model the newly released planktonic cells are washed downstream by the continual flow of media were they can be collected and studied. Studies assessing the characteristics of cells collected following a dispersal event report different phenotypic and morphological characteristic to those of the parental strain (Kirov *et al.* 2007; Woo *et al.* 2012; Workentine *et al.* 2010). Dispersed cells collected from the current study also possessed different colonial morphologies compared to those initially inoculated into the flow cell chambers. When compared to the environmental isolates, the AUST-02 strains also generated significantly greater numbers of dispersed cells. To determine whether the increased number of dispersed cells was influenced by the mass or surface area of the biofilm, repeat analysis, adjusting for these parameters was performed. This analysis demonstrated that biomass of the biofilm was indeed a major contributing factor of cellular dispersal. These data are supported by earlier work from Purevdorj-Gage and colleagues who also showed that microcolony size and mass correlates with hollowing and dispersal events (Purevdorj-Gage *et al.* 2005). It is therefore tempting to speculate that a combination of increased biofilm biomass and cellular dispersal could be important for person-to-person transmission and niche adaptation of the AUST-02 strain within the CF airway microenvironment.

To comment on the impact of infection duration and how chronicity influences biofilm formation and maturation, *P. aeruginosa* collected from two patients were analysed over a 12-year period. Unfortunately, neither patient had isolates retained from the time of initial infection, therefore a representative of an early infecting strain could not be included in this analysis. Regardless, this current study revealed no marked difference in either mass or structure of the biofilm over the observational period. Furthermore, the phenotypic characteristics of these isolates did not alter over time, remaining non-motile and non-adherent under static conditions. These results contrast those of Lee and colleagues, who demonstrated that as infection duration progresses there is a corresponding reduction in the organism's ability to adhere to the glass coverslip and form a biofilm within a continuous flow cell model (Lee *et al.* 2005). Interestingly, this decline in biofilm development corresponded to the loss of motility for these strains.

Although this study was thorough in the investigation of adhered biofilm development, it did not assess the presence of bacterial cluster formation. Studies have identified bacterial clusters within

sputum samples from patients with CF (DePas *et al.* 2016). The presence of varied phenotypes within the airways may contribute to the enhanced persistence and poor efficiency of eradication therapies, but due to experimental design this feature could not be identified or studied.

There are a number of limitations to the current study, both regarding the isolate selection and the protocol design. Firstly, due to the time taken to run each experiment and the complexity of this study design it was only feasible to assess biofilm development on a modest number of isolates. In addition, whilst the clinical isolates all belonged to the same genotype and were obtained from the airways of people with CF, the environmental isolates represented six unique sequence types. Consequently, it is possible that the variance in genotype and collection site may have played a role in these findings. Secondly, as previously stated, the initial or early infecting isolates were unavailable for testing, so longitudinal isolates collected many years after the onset of the chronic infection were analysed. Therefore, specific mechanistic changes which may occur during the period between initial acquisition and the onset of chronic infection could not be determined and any inference of phenotypic characteristics from this work must be interpreted with caution. Thirdly, due to limitations regarding protocol design the daily monitoring of the developing biofilms using the CLSM could not be undertaken. Hence, specific details regarding developmental stages and dispersal events were not included in analysis. To address this, further work is planned to more accurately monitor dispersal events of both environmental and clinical isolates. Fourthly, this study did not perform any i) microscopy to determine the presence or absence of cell surface structures such as pili or flagella, ii) genetic analysis to identify mutations recognised to control motility, adherence or biofilm development and iii) expression studies to confirm the effects of the mutations. However, whole genome-based studies are now planned with the aim of providing additional mechanistic insights. Finally, results generated from *in vitro* testing may not accurately represent *in vivo* conditions. Under these experimental conditions there were no fluctuations in nutrient content, oxygenation, temperature or exposure to a range of antimicrobial substances, whereas in the human host or within some natural environmental settings the bacteria would be exposed to a range of fluctuating conditions, numerous other organisms and many antimicrobial substances. So, while these results reflect the conditions under which they are tested, caution must be applied when extrapolating into clinical situations. Due to experimental design, it was not feasible to demonstrate an association between the capacity to form biofilms and the clinical impact caused by infections with these strains. However, these results suggest that despite the downregulation of many traits required to form biofilms, the AUST-02 strains retain this capability. Therefore, it is hypothesised that other mechanisms which regulate biofilm formation are present in these bacteria. Clinically, these strains

would demonstrate a greater resistance to anti-pseudomonal antibiotics and enhance persistence, leading to poor prognosis for patients with an infection.

Overall, the results from study show that despite similar levels of adherence, as demonstrated by homogeneous monolayer development on the glass coverslip within the continuous flow cell model, the AUST-02 strain, formed thicker biofilms consisting of well-developed cellular structures, compared to environmental isolates. Furthermore, results obtained from high throughput methodologies, such as a static microtitre plate-based model cannot be used to infer behaviour within a continuous flow cell model. Therefore, caution must be shown when i) choosing a method to assess *in vitro* biofilm formation ii) interpreting results and iii) inferring *in vivo* capabilities of a *P. aeruginosa* strain. Together with existing work, this study highlights the adaptive nature of *P. aeruginosa* and lends further weight to the importance of understanding the mechanistic differences which underpin persistence and infection.

Chapter 6: Genomic analysis of *Pseudomonas aeruginosa*

6.1 Abstract

Background: Due to the high prevalence and association with adverse clinical outcomes, the presence of the bacterium *Pseudomonas aeruginosa* in the airways of people with cystic fibrosis (CF) is highly clinically relevant. During the establishment of chronic respiratory infections in people with CF, *P. aeruginosa* evolves specific characteristics that make this pathogen better adapted to the lung environment. Many previous studies have highlighted the complexity of this process and currently the molecular basis underpinning many of these phenotypic adaptations remain poorly understood. Therefore, using a comparative genomic approach this study aims to identify the genetic basis of mechanisms key to airway adaptation.

Methods: Thirty *P. aeruginosa* isolates obtained from either patients with CF or the local environment underwent phenotypic testing to determine motility and adhesion capabilities. Following this, whole genome sequencing and bioinformatic analysis was performed on all isolates. To assess correlation between phenotypic characteristics and genome, two analyses were performed; genome wide association studies (GWAS) and genome mapping and assembly to allow for the visualisation of specific variants within each isolate.

Results: GWAS identified a number of genetic variants which may affect the phenotypic capabilities of these isolates. Closer examination of these variants within each of the isolates showed that while a number of these mutations were highly correlated with the loss of function, no single variant could fully account for the phenotype expressed.

Conclusion: Rather than identifying a genetic variant responsible for these phenotypic characteristics this study has confirmed that the mechanisms responsible for specific bacterial traits (including motility and adhesion) are complex and multifactorial. This combined approach has highlighted a number of genes, which would be good targets for future analysis using a functional approach to assess the genetic variant's contribution to phenotype.

6.2 Introduction

Pseudomonas aeruginosa is the leading cause of respiratory infections in people with cystic fibrosis (CF) (Cystic Fibrosis Australia 2016; Cystic Fibrosis Foundation 2015; Cystic Fibrosis Trust 2016). Importantly and of clinical relevance, individuals with CF that become chronically infected with *P. aeruginosa* have significantly poorer health outcomes than non-infected individuals (Lee *et al.* 2003; Vidya *et al.* 2016). When a patient first becomes infected with wild-type *P. aeruginosa* the bacterium is not necessarily well adapted to life in the CF lung. Over time, the pathogen evolves and adapts to the lung environment generally transitioning from a motile free-floating planktonic cell into a non-motile cell living within a complex bacterial community, known as a biofilm (Cullen and McClean 2015; Sousa and Pereira 2014; Winstanley *et al.* 2016). Growth within a biofilm community offers several advantages, including increased tolerance to antibiotic treatment and physical protection from external environmental factors (Costerton *et al.* 1995). Multiple studies have attempted to identify candidate genes that exhibit altered expression profiles or mutational adaptation as the bacterium becomes better suited to the lung environment. Many such screening studies have identified genes essential for motility or biofilm formation, an observation which have been confirmed by the construction of knockout strains (Dasgupta *et al.* 2003; Hammond *et al.* 2015; Marvig *et al.* 2013; Marvig *et al.* 2015; Mattick *et al.* 1996; Mena and Gerba 2009; Oliver and Mena 2010; Semmler *et al.* 2000). However, rather than the identification of single genes or gene variants responsible for specific phenotypic changes, these studies have suggested that these mechanisms are complex and multifactorial.

Following whole genome sequencing (WGS) and bioinformatic analysis; two differing approaches can be used to identify candidate genes associated with phenotypic characteristics. Firstly, a targeted approach, based on a literature search to identify genes known to effect protein function resulting in altered phenotype can be performed, followed by read mapping and sequence alignment identifying nucleotide substitutions within these candidate genes. The predicted effect of the amino acid sequence variation can be determined and results visualised. This methodology is limited as only genes currently known to be associated with phenotype may be analysed (Sherrard *et al.* 2017).

Alternatively, genome-wide association studies (GWAS) to identify genetic variants correlated with disease. A GWAS is an unbiased, statistically based screening method that can determine if genetic variants, such as single nucleotide polymorphisms (SNPs), insertions and deletions (indels) or entire genes are over-represented within a specific population possessing a phenotypic trait compared with matched controls (Bush and Moore 2012; Corvin *et al.* 2010; Read and Massey 2014). While this approach has been readily used to identify genetic polymorphisms associated with specific inherited

genetic disease in humans (Hardy and Singleton 2009; Manolio 2010), only recently has this technique been applied to bacterial genomics to assist in further determining the mechanisms associated with resistance, transmission and adaptation (Chen and Shapiro 2015; Power *et al.* 2017; Read and Massey 2014).

Results from Chapter 4 demonstrated that *P. aeruginosa* obtained from the environment are phenotypically distinct from clinical strains obtained from patients with CF. As isolates from these studies fall within two defined groups (e.g. motile versus non-motile), this collection is suitable for GWAS style approach to identify genetic variants, which may be responsible for these phenotypes. Furthermore, existing studies have highlighted many target genes which may affect protein function, which can be rapidly screened for genetic variants.

Although *P. aeruginosa* has been extensively studied the molecular basis of many phenotypes remains poorly characterised and based on previous results, it is difficult to associate a given mutation with a specific phenotypic characteristic; however, using a well characterised selection of isolates and by performing thorough genomic analyses, it is hypothesised that mutations known to effect protein function will be identified. Therefore, this chapter comprehensively characterises three motility phenotypes (swarming, swimming and twitching) and bacterial adhesion, under three atmospheric conditions (aerobic, microaerophilic and anaerobic) in 30 *P. aeruginosa* strains. These isolates were then subjected to WGS and large-scale comparative genomics analysis to identify the genetic basis of motility and adhesion in this collection.

6.3 Materials and Methods

6.3.1 Isolate Selection

Based on phenotypic characteristics determined in Chapter 4, isolate selection included environmental isolates which displayed enhanced motility and adhesion, as well as CF clinical strains which showed impairment of these phenotype characteristics. Overall, 30 *P. aeruginosa* strains were selected for whole genome sequencing and analysis comprising13 AUST-02, five AUST-06 and one unique strain isolated from respiratory samples of people with CF with a chronic infection and 11 isolates obtained from the natural environment (Kidd *et al.* 2013; Kidd *et al.* 2012) (Table 6.1).

6.3.2 Isolate storage, retrieval, identification and genotyping

Refer to sections 3.3.2 to 3.3.6 from Chapter 3 and sections 4.3.1 to 4.3.6 from Chapter 4 for isolate handling, preparation and identification techniques used in this Chapter. Twenty-two isolates underwent multilocus sequence typing (MLST) to confirm their genotype, while SNP typing was performed on the remaining eight isolates. *In silico* MLST analysis of the eight isolates that underwent SNP typing confirmed that four isolates belonged to ST-775 (AUST-02), three were confirmed as ST-801 (AUST-06), and one isolate (AUS883) comprised a novel double-locus variant (DLV) of ST-801 (Table 6.1).

6.3.3 Phenotypic Assays

All methodologies and interpretation are described in section 4.3.7 from Chapter 4. Results from media based motility assays and microtiter plate-based model obtained from Chapter 4 (Table 4.5) are presented for all isolates listed in in Table 6.1.

Table 6.1 Complete list of isolates used for phenotypic testing and genomic analysis.

WGS Number	Source	Isolate source	Sequence Type
AUS276	Environmental	Water	9
AUS465	Environmental	Water	147
AUS438	Environmental	Water	155
AUS502	Environmental	Swab: Air/Water Interface	179
AUS222	Environmental	Swab: Air/Water Interface	209
AUS227	Environmental	Water	216
AUS343	Environmental	Water	266
AUS306	Environmental	Water	381
AUS504	Environmental	Swab: Air/Water Interface	389
AUS503	Environmental	Swab: Air/Water Interface	810
AUS449	Environmental	Water	930
A2_08	CF Patient (Adult)	Sputum	775
A2_07	CF Patient (Adult)	Sputum	775
A2_04	CF Patient (Adult)	Sputum	775
A2_29	CF Patient (Adult)	Sputum	775
AUS727	CF Patient (Adult)	Sputum	775
A2_06	CF Patient (Adult)	Sputum	775
A2_11	CF Patient (Adult)	Sputum	775
A2_13	CF Patient (Paediatric)	Sputum	775
A2_18	CF Patient (Paediatric)	Sputum	775
A2_19	CF Patient (Paediatric)	Sputum	775
A2_41	CF Patient (Adult)	Sputum	775
AUS717	CF Patient (Adult)	Sputum	775
A2_43	CF Patient (Paediatric)	Sputum	775
AUS883	CF Patient (Adult)	Sputum	Novel ST*
AUS77	CF Patient (Paediatric)	Sputum	801
AUS884	CF Patient (Adult)	Sputum	801
AUS885	CF Patient (Adult)	Sputum	801
AUS396	CF Patient (Adult)	Sputum	801
AUS886	CF Patient (Adult)	Sputum	801

* Double-locus variant to ST-801.

6.3.4 DNA extraction

Genomic DNA was prepared using the UltraClean® Microbial DNA Isolation Kit (Mo Bio) with the following modifications to the protocol. Prior to extraction, samples were cooled on ice for 1 hour, pelleted at 14,000 x *g* for 1 minute and washed twice using 0.9% Sodium chloride at 14,000 x *g* for 1 minute. To increase DNA yield each sample was heated at 70°C for 10 minutes and bump vortexed for 15 seconds at 2 minute intervals. During precipitation of the non-DNA material

the samples were cooled on ice for 10 minutes and mixed gently. Centrifugation speed and time was increased to 12, 000 x *g* and 1 minute during the DNA binding steps and the final elution volume was increased to 100 µL.

Genomic DNA quantity and concentration was determined using NanoDrop Spectrophotometry (Thermo Scientific Inc.). Library preparation and WGS were carried out at either the Australian Genomic Research Facility (AGRF), Melbourne, Australia or at the University of Laval, Québec, Canada using the Illumina HiSeq 2500 platform and Illumina MiSeq instrument, respectively (Sherrard *et al.* 2017).

6.3.5 Variant identification and genome wide association analysis

Genome wide association studies were performed using the SPANDx pipeline (Sarovich and Price 2014) and PLINK (Purcell *et al.* 2007). *P. aeruginosa* strain PAO1 was used as the reference genome (Accession number: NC_002516) (Stover *et al.* 2000). Variants including both SNPs and indels were identified and annotated using BWA for read alignment (Li and Durbin 2009), SAMtools for read filtering and parsing, Picard (GitHub Inc Picard) for data filtering, the Genome Analysis Tool Kit (GATK) for base quality score recalibration, variant determination, data filtering and improved insertion-deletion calling, VCFtools for SNP and indel matrix construction, and SnpEff for variant annotation. Variants were then tested for association with phenotypes using PLINK (Figure 6.1).

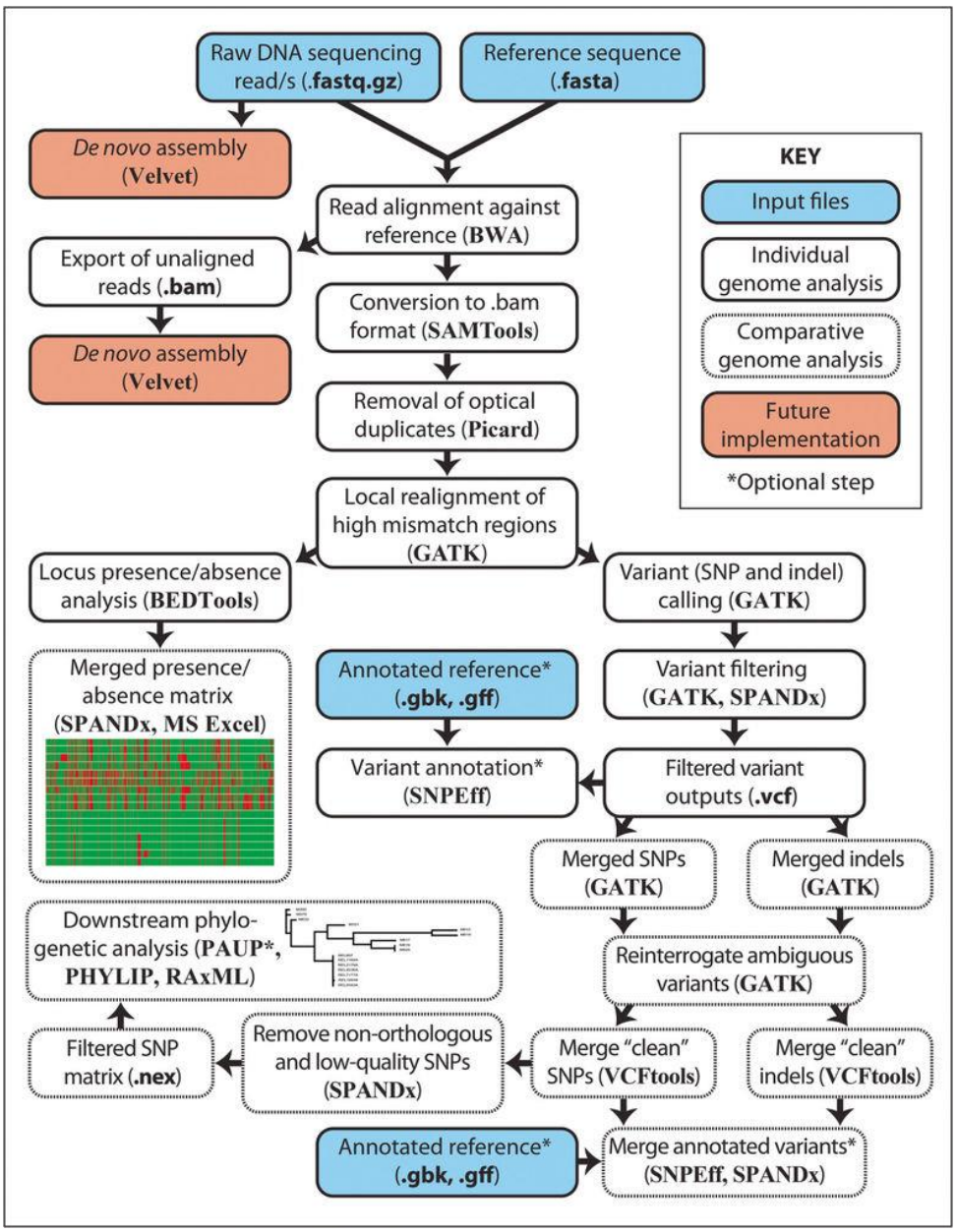


Figure 6.1 Genome wide association workflow.

6.3.6 Genome mapping and assembly

In parallel with the GWAS analyses each assembled and annotated genome was compared with the PAO1 reference genome to identify genetic differences and functionally important mutations. To this end, Kraken (v0.10.4; (Wood and Salzberg 2014)) was used to compare the whole genome sequencing reads to known *P. aeruginosa* sequences to confirm genus and species and identify any DNA considered to be contamination. Nesoni (v0.128; (GitHub Inc Nesoni) was then used to filter out adapter sequences and low quality regions within the whole genome sequencing reads. SHRiMP2 (David *et al.* 2011), as implemented within the Nesoni toolset, was thereafter used to map the reads to the PAO1 genome (Stover *et al.* 2000). SNPs (leading to synonymous (SMs) or non-synonymous mutations (NSMs) or premature stop codons) and indels were called using Nesoni. Ambiguous calls

were also clearly highlighted and were not included in the final analysis. In parallel to mapping, *de novo* assembly was also performed using Velvetoptimiser (v2.2.5; (GitHub Inc VelvetOptimiser)) and Velvet (Zerbino and Birney 2008) to assemble the whole genome sequencing reads. Assembled contigs were reordered against PAO1 using Mauve (v.2.4.0; (Darling *et al.* 2010)) and annotated using Prokka (v1.10; (Seemann 2014)). Gene annotations from PAO1 were used as the primary reference (Sherrard *et al.* 2017). Finally, a BLAST (Basic Local Alignment Search Tool) alignment of all predicted coding regions (using the genome assemblies generated) against the panel of genes of interest was performed to identify large genetic differences between the AUST-02, AUST-06 and environmental isolates and PAO1. When the nucleotide identity of a target gene was ≥97% (compared to PAO1), then any variants called by Nesoni were manually inspected. Functionally important mutations were defined as premature stop codons or frame-shift mutations. The effect of amino acid changes on protein function (effect or no effect) can be predicted using computational algorithms (Prickett *et al.* 2017). PROVEAN (Protein Variation Effect Analyzer) was used to filter the remaining missense and in-frame mutations into those that were predicted to be functionally important or neutral using the default score thresholds (Choi and Chan 2015; J Craig Venter Institute).

6.4 Results

6.4.1 Motility in *P. aeruginosa* varies considerably across a diverse strain collection

Thirty strains were tested for their ability to swim, swarm and twitch in different atmospheric conditions. Regardless of the atmospheric conditions, the clinical strains displayed reduced motility compared to isolates from the environment. Only 6% and 31% of all the motility tests performed returned a positive result for the AUST-02 and AUST-06 strains, respectively. Despite displaying substantial phenotypic heterogeneity, 81% of all motility assays performed on isolates obtained from an environmental origin showed evidence of motility (Figure 6.2a – i).

6.4.2 Environmental isolates display enhanced adhesion capabilities

All of the environmentally-derived isolates displayed adhesion capabilities in the static microtitre plate-based assay under each atmospheric condition. In contrast, only three AUST-02 isolates and none of the AUST-06 strains showed adhesion in this model (Figure 6.2j – l).

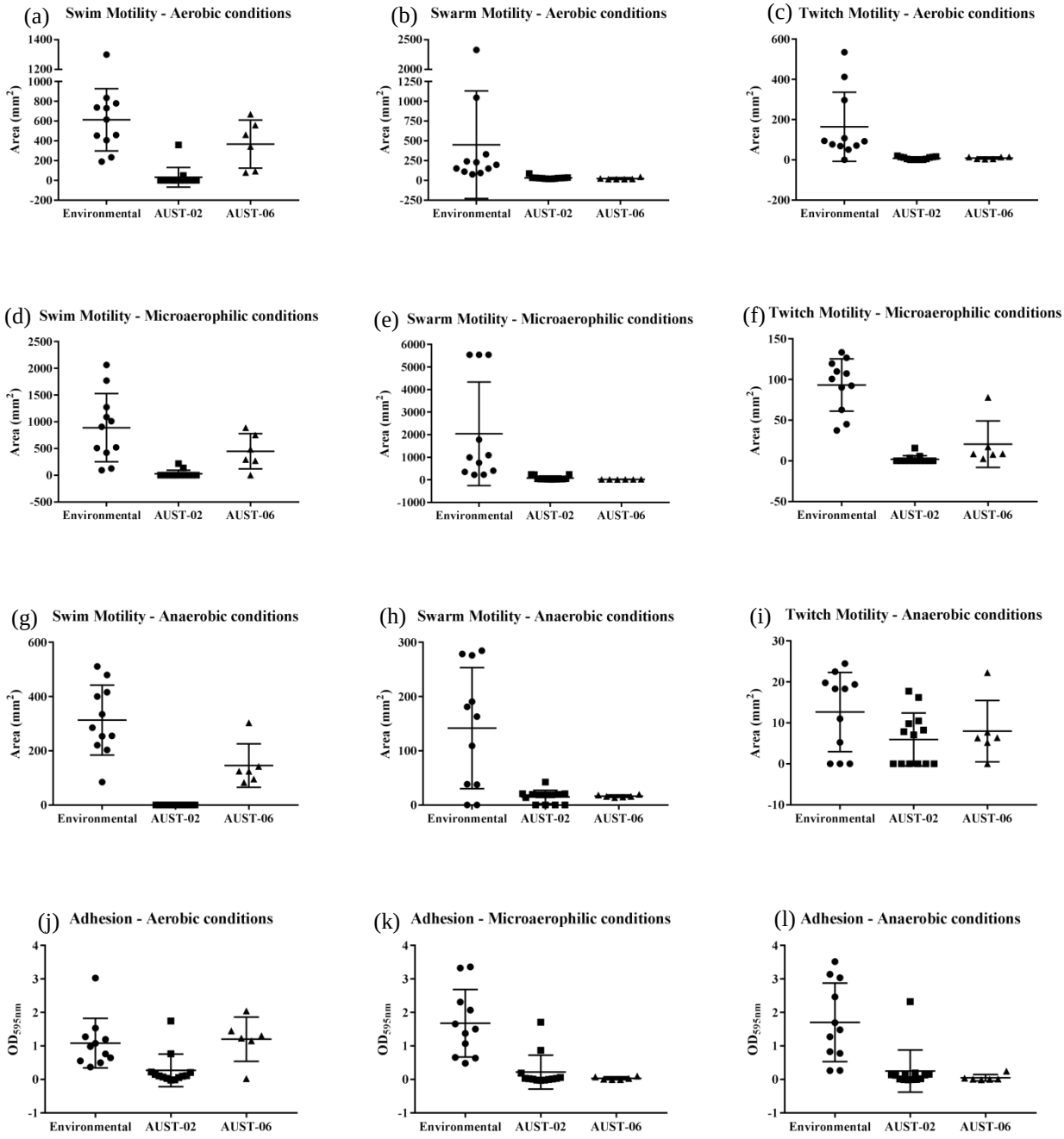


Figure 6.2 Phenotypic testing of multiple *P. aeruginosa* isolates from environmental (n = 11) or clinical sources (AUST-02 strain, n = 13; AUST-06 strain, n = 6).

Tested phenotypes include (a) swim, (b) swarm and (c) twitch motilities in aerobic conditions; (d) swim, (e) swarm and (f) twitch motilities in microaerophilic conditions; (g) swim, (h) swarm and (i) twitch motilities in anaerobic conditions; adhesion results from the microtitre plate-based assay using LB media in (j) aerobic, (k) microaerophilic and (l) anaerobic conditions. The average motility result was tested in triplicate and six replicates from the microtitre assay are presented, with error bars representing the mean and standard deviation.

Note: Adapted from data presented in Chapter 4. Subset analysis of isolates undergoing WGS.

6.4.3 Phylogenetic reconstruction shows that there is little within ST variation

Phylogenetic reconstruction using 115,494 genome-wide orthologous SNPs showed that all the AUST-02 isolates were very closely related showing negligible within sequence type (ST) variation. Likewise, the AUST-06 and AUS883 DLV isolates formed a tightly clustered phylogenetic clade (Figure 3). In comparison, the environmental isolates showed greater diversity and were separated by considerable genetic distance from the AUST-02 and AUST-06 clades. Mapping of the phenotypic data against the phylogenetic tree showed that there was considerable population bias for some phenotypes. The most extreme example was observed with the anaerobic swimming phenotype being completely absent in the AUST-02 clade and present in all other isolates.

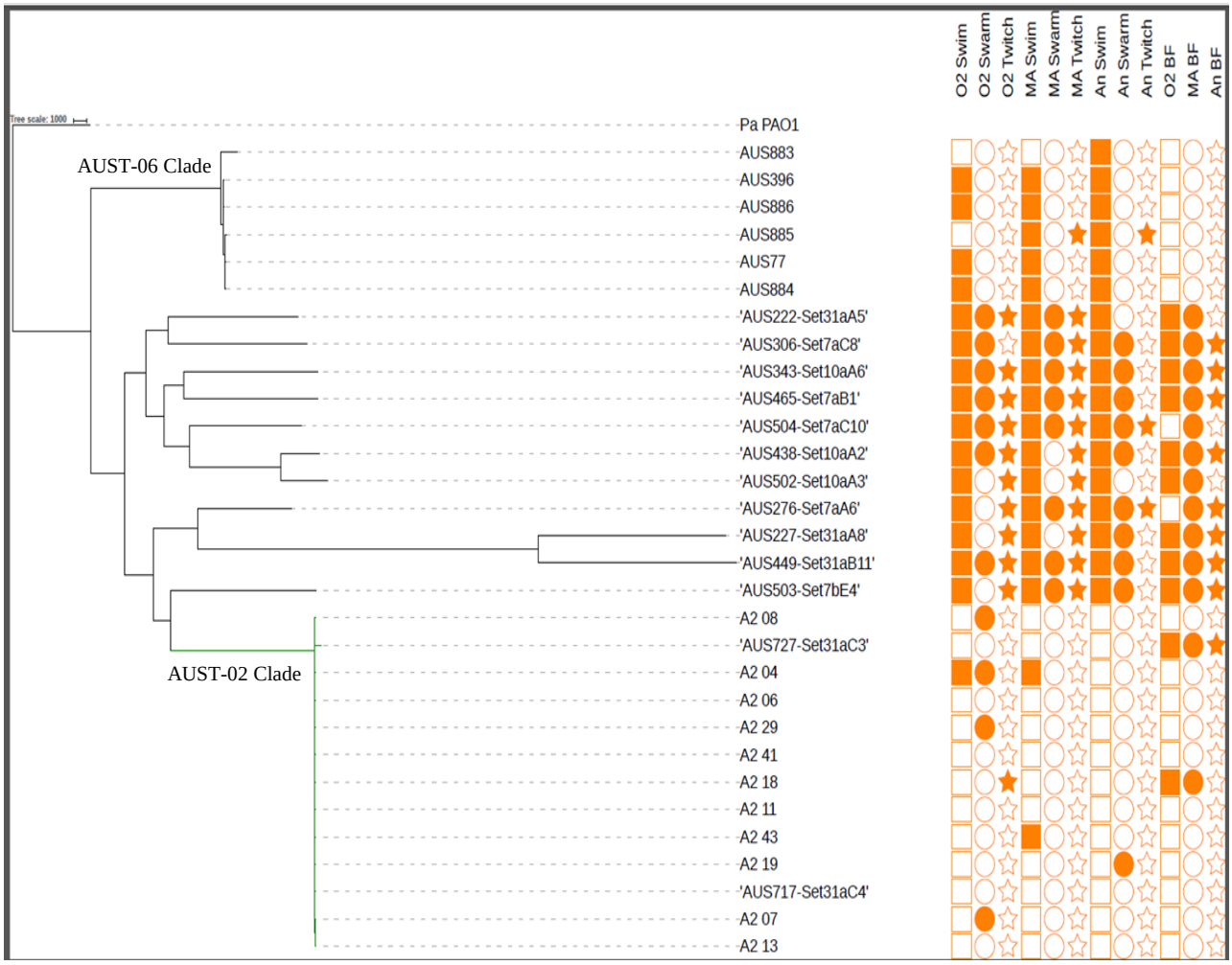


Figure 6.3 Phenotypic trait data correlated with maximum-parsimony phylogenetic reconstruction using 115494 genome-wide SNPs.

Solid shapes represent a functional phenotype.

Abbreviations: BF, biofilm formation (adhesion); O_2, aerobic; MA, microaerophilic; An, anaerobic.

6.4.4 GWAS

All SNPs and indels were identified, annotated and tested for correlation with specific phenotypes. The most significant associations (up to five) between genotype and phenotype are presented in Appendix 5.1. Several candidate mutations were identified that were significantly associated with the majority of phenotypes (Table 6.2). Results for specific phenotypes are presented below.

6.4.5 Genome mapping and assembly

Using the results generated from the GWAS analysis, genome mapping and assembly was performed to identify nucleotide substitutions from the candidate genes displaying strong correlation with the phenotypes described (Table 6.3).

6.4.6 Swim Motility

6.4.6.1 GWAS

No single genetic variant explained a negative swim motility phenotype within this sample set; however, several strongly associated variants were identified through GWAS analysis and included putative enzymes, hypothetical proteins, outer membrane proteins associated with alginate production, efflux pumps and iron sequestration determinants. Interestingly, the ten most significant genetic variants were very similar for aerobic and microaerophilic swimming motilities (Appendix 5.1). Two of these indel variants, *aprE* and *pscK*, have been previously been associated with bacterial motility (Table 6.2). Unfortunately, under anaerobic conditions, genetic variants underpinning swimming motility were not able to be identified due to severe population stratification that phenotypically and genetically separated the AUST-02 group from all other strains (Figure 6.3). Visualisation of this population bias is presented in the GWAS Manhattan Plot below (Figure 6.4).

6.4.6.2 Genome mapping and assembly

Analysis of this sample set showed that the AUST-02 *aprE* gene possessed an indel at -78 base-pairs (bp) upstream of the start site, which may affect expression of this gene leading to reduced motility. Likewise, the AUST-02 *pscK* gene contained an in-frame deletion (Cys152del) which was predicted to alter its function. Comparisons to phenotypic results confirmed that both of these indels appeared to be very strongly correlated with swimming motility under each atmospheric condition. Excluding two phenotypic outliers (A2_04 and A2_43), the presence of these indels appears to impair swimming motility, whereas, 15/17 of the AUST-06 clade and environmental strains in which these mutations not identified were capable of swimming motility across the various atmospheric conditions. Interestingly, complete correlation was noted for anaerobic swimming; however, this could not be corroborated by GWAS analysis. In addition, all of the AUST-02 and AUST-06 clade strains contained other deleterious mutations within the *aprE* (E75V) and *pscK* (RRL161_163R) genes.

However, each of these variants did not appear to impede swimming motility as all but one of the ST-801 strains carried this mutation and retained positive aerobic, microaerophilic and anaerobic swimming phenotypes. Notably, the environmental isolates showed high level homology with the PAO1 *aprE* and *pscK* sequences which corresponded with a positive swimming motility phenotype in all instances (Table 6.3). Finally, GWAS analysis of aerobic and microaerophilic swimming identified a duplication within the PA1874 gene (Ser150_Gly151dup). This gene has previously associated with resistance mechanisms of bacteria comprising biofilm structures, but given that 100% of the isolates showed low nucleotide identity to PA01 we were unable to determine if duplication of this loci may also impact on swimming motility.

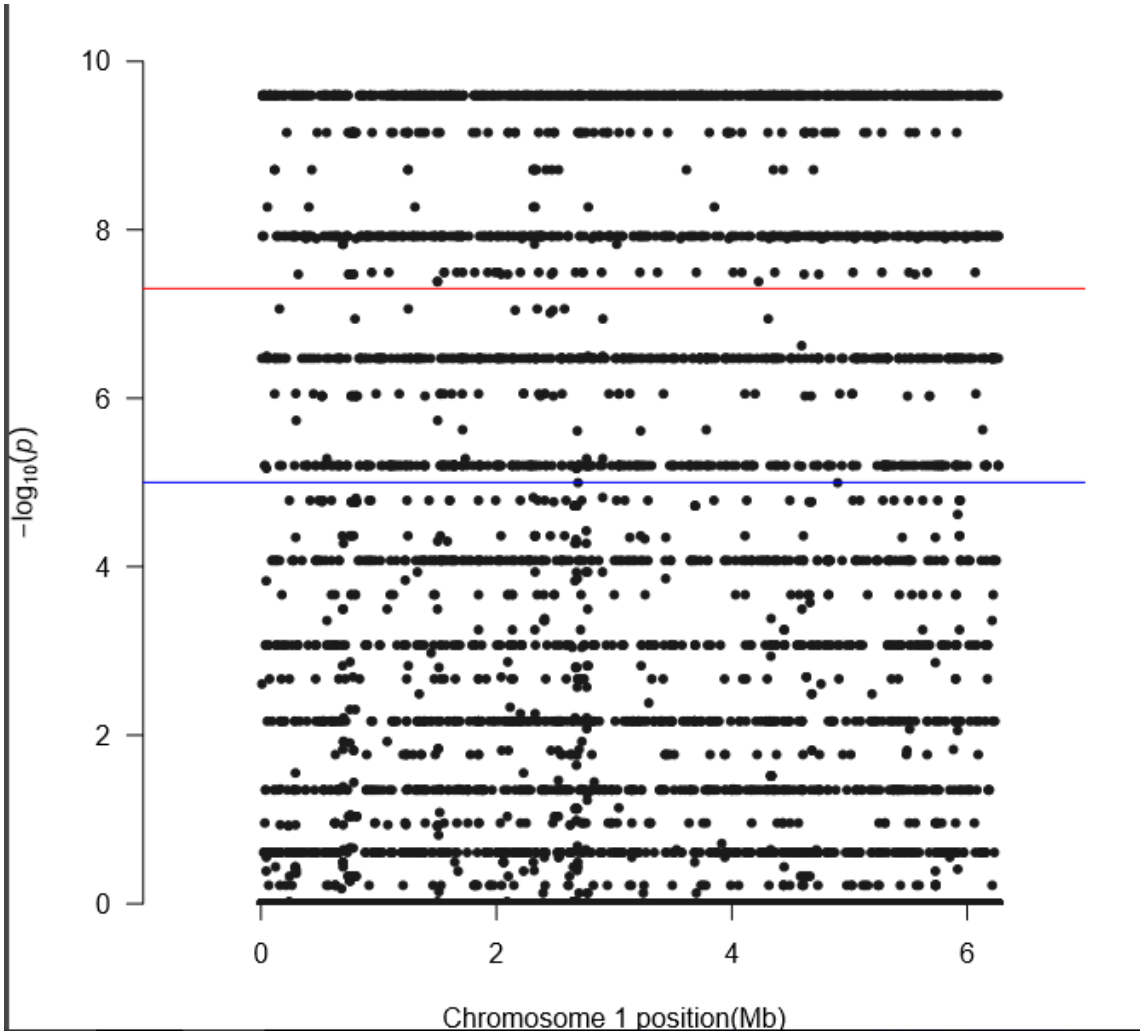


Figure 6.4 Manhattan plot showing population stratification impeding the identification of genetic variants correlating with the anaerobic swimming motility.
Red and blue lines represent significance cutoffs 1 x 10^{-5} and 1 x 10^{-7} respectively.

6.4.7 Swarm Motility

6.4.7.1 GWAS

Presented in Appendix 5.1 are the top four genetic variants identified by GWAS analysis for swarming motility under aerobic conditions. This analysis did not identify any indels associated with aerobic swarming. No correlation was observed when these four variants and the phenotypic characteristics displayed by all isolates were compared. When this analysis was performed on the isolates under reduced oxygen environments, a number of different variants demonstrating high levels of correlation were identified. Indeed, the presence of these variants was able to differentiate the swarm negative AUST-02 and AUST-06 isolates from the swarm positive environmental isolates. These variants include a number of hypothetical proteins, enzymes and an outer membrane efflux system protein (Appendix 5.1). Two SNP variants within PA1908 and PA4881, were shown to correlate completely with swarm motility under microaerophilic conditions and three SNP variants, within PA0209, PA1997 and *hpaA*, were highly correlated with swarm motility under anaerobic conditions (Table 6.2). Furthermore, GWAS analysis did show that the genes *flgK*, *fimV*, *xcpQ* and *clpB* to be associated with swarming in reduced oxygen environments (Table 6.2).

6.4.7.2 Genome mapping and assembly

The genes which were highly correlated with phenotype and those previous associated with motility and adhesion identified during GWAS analysis were more closely examined using mapping and assembly techniques. These analyses showed that compared to the reference PAO1 strain, a number of isolates displayed low nucleotide identity to the highlighted genes. For example, 11 of the AUST-02 isolates displayed less than 50% nucleotide identity with the PAO1 PA1908 gene sequence. Likewise, 100% of the clinical strains and four environmental isolates (AUS222, AUS438, AUS502, AUS504) demonstrated low sequence homology with the PAO1 PA4881 gene. In each of these instances this sequence variation was predicted to have functional consequences on protein synthesis and/or function.

This analysis also revealed that the majority of variants identified by the GWAS analyses comprised synonymous mutations (SMs), which in some cases correlated strongly with a lack of swarming motility (Table 6.3). Indeed, the G91A PA1908 gene SM was detected in 11 isolates (including AUST-02, AUST-06 and environmental), nine of which were negative for microaerophilic swarming motility. Similarly, the SM *clpB* V846L variant was also highly correlated with negative swarming under microaerophilic conditions. Other examples included PA1997 G215D and *hpaA* gene I477V variants which both often correlated with a non-swarming phenotype in anaerobic conditions. Overall, we identified only one gene showing a loss-of-function mutation (i.e. *fimV* Gly682_Asp683insGlyAspLeuGlySer) among variants detected by GWAS analyses of the swarming motility phenotype. This indel was present in all but one (A2_19) of the clinical isolates, and two

environmental isolates (AUS227, AUS449). Phenotypic comparison confirmed a very strong correlation in microaerophilic or anaerobic conditions.

6.4.8 Twitch Motility

6.4.8.1 GWAS

GWAS did not identify any variants to be significantly correlated with twitching under anaerobic conditions and due to population bias no SNPs were identified when twitching was assessed microaerophilically. Nevertheless, GWAS identified SNP variants within PA4781, PA4782, PA5484, PA5492 and PA0683 that were significantly correlated with loss of twitch motility in aerobic conditions. While this analysis identified a number of indels, none displayed a high correlation with phenotypic traits, however indels within *xcpQ*, *fimV* and *pilQ* genes, previously associated with motility and adhesion were identified following GWAS (Table 6.2, Appendix 5.1). As demonstrated in Figure 6.5, the indel in *xcpQ* was highly correlated with twitching under microaerophilic conditions for this data set. Other variants were identified through GWAS analysis, but are not currently known to influence aerobic twitching motility, included frameshift mutations associated with antibiotic tolerance, iron sequestration and cell wall biosynthesis, as well as hypothetical protein and an enzyme associated with carbon utilisation (Appendix 5.1).

6.4.8.2 Genome mapping and assembly

Assessment of genetic composition determined that with the exception of the A2_18 strain, mutations within PA4781 (L158F), PA4782 (A6V), PA5484 (E54K), PA5492 (V191I) and PA0683 (E240G) genes correlated strongly with the loss of a twitching phenotype. However, each of these SNPs were not predicted to result in deleterious mutations. Interestingly, while both AUST-02 and environmental strains share less than 90% nucleotide identity with the *pilQ* gene from PAO1, the clinical strains were almost entirely twitch negative while the converse was true for the environmental strains. The *fimV* gene was also highly heterogeneous with numerous indels noted mainly across the clinical isolates. Consequently, it is difficult to correlate any one variant with a particular phenotype and given the population stratification of the dataset parallel evolution is highly likely to have occurred as these respective populations have adapted to their host. Nevertheless, it is interesting to note that the *fimV* Gly682_Asp683insGlyAspLeuGlySer indel which was associated with loss of swarming motility, strongly correlated with a lack of twitching motility, particularly under microaerophilic conditions (Table 6.3).

All the clinical strains and two environmental strains (AUS227, AUS449) demonstrated low nucleotide identity with the *xcpQ* sequence of the PAO1 strain, which was not seen within the environmental cohort Targeted analysis of the remaining environmental strains to determine the

presence of the *xcpQ* indel as identified by GWAS (Glu417_Ser418delinsAla) did not identify this variant in any strains. Therefore this variant is not fully associated with loss of motility (Table 6.3).

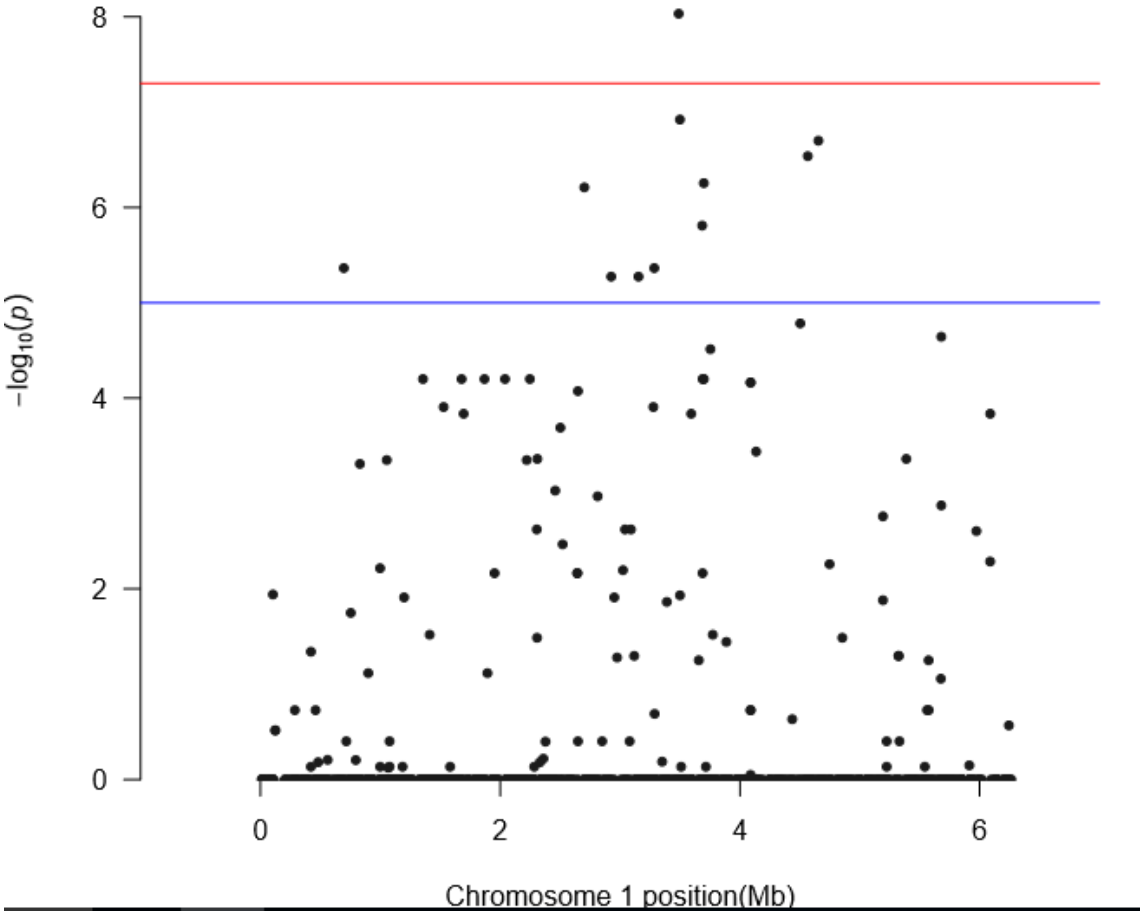


Figure 6.5 Manhattan plot for microaerophilic twitching motility.
Red and blue lines represent significance cutoffs 1 x 10^{-5} and 1 x 10^{-7} respectively. The one variant above the red line is an indel within *xcpQ* (Glu417_Ser418delinsAla).

6.4.9 Adhesion and initial biofilm formation

6.4.9.1 GWAS

GWAS analysis was successful when identifying both SNPs and indels for adhesion under each of the atmospheric conditions (Appendix 5.1). Many different variants associated with bacterial adhesion were identified, including those involved with antibiotic tolerance, secretion systems, iron sequestration, bacterial communication and genetic recombination. Included were a number of variants previously associated with motility and adhesion phenotypes. GWAS identified three SNPs in PA2069, PA3292 and *gcvP*1, to be functionally important during aerobic bacterial adhesion (Table 6.2, Appendix 5.1), and a further two SNP variants within PA0144 and PA2125, which may play a role during bacterial adhesion under reduced oxygen environments. Informative indels in four genes (*opr*86, *pilQ*, *xcpQ*, and PA4156) were also identified (Table 6.2).

6.4.9.2 Genome mapping and assembly

Each of the genes identified by GWAS demonstrated a high degree of sequence heterogeneity, with none correlating completely with the displayed phenotypes. Poor nucleotide identity with the PA0144 was noted for all the clinical strains and five of the environmental isolates. Of those isolates with a low nucleotide identity to PAO1, this correlated with non-adherence for all of the AUST-06 isolates, 77% of the AUST-02 isolates and did not infer non-adherence in the environmental isolates. The PA2125 gene had a number of different SNPs though none were associated with a loss of function (Table 6.3). However, the SM identified by GWAS (K67T) was found in a half of the adhesion-positive environmental isolates and absent from all other isolates. Similar results were found when the SMs from PA2069 (A480E), PA3292 (S202P) and *gcvP1* (S98P) genes, none could be used to determine phenotypic traits. A six bp deletion (PMK674_676Q) in the *opr86* gene was identified in all the AUST-02 strains, 77% of which were non-adherent in both aerobic and microaerophilic conditions. This deleterious mutation was not noted in any other isolate. The two *opr86* indels (Arg668FS, Thr673_Met675del) identified by GWAS, were not shown to demonstrate a high level of correlation with loss of adhesion; however, this analysis was hampered by poor sequence quality in many of the strains. Interestingly, the environmental isolates comprised the majority of isolates in which these variants were identified. As each of these strains displayed strong adhesion capabilities, it is apparent that these variants do not have a substantial impact on cellular adhesion. Both the AUST-02 and environmental strains had very large genetic variations within *pilQ* compared to PAO1; however, phenotypically these strains displayed contrasting morphologies. Furthermore, all AUST-06 strains were non-adherent but possessed a *pilQ* SM (A106T) that was not predicted to effect protein function. All the clinical isolates displayed low nucleotide identity with PAO1 for the *xcpQ* gene. Although the majority of the clinical strains were non-adherent, the two environmental isolates with this genetic variation still displayed bacterial adherence when using a static biofilm model (Table 6.2). Finally, the presence of a number of deleterious mutations and a frameshift mutation (Gly471FS) was identified in PA4156, although none correlated completely with a loss of adhesion capability within this isolate collection (Table 6.3).

6.4.10 Other mutations associated with phenotypic traits

Using mapping and assembly tools to more closely assess the genomes of the isolates tested has demonstrated that a number of variants can be associated with phenotypes, different to those identified in the GWAS analysis (Table 6.3).

The single bp deletion (C152del) identified in the *pscK* gene appeared to be more strongly correlated with anaerobic swimming than swimming under aerobic or microaerophilic conditions. The mutations, E75V and A168S (*aprE* gene) and the 6 bp deletion in *pscK* displayed greater synergy with a loss of adhesion and twitch motility, respectively than swim motility. Further work is needed but from this analysis, it appears that a number of NSMs and SMs within the *opr86* gene may functionally impact anaerobic swimming, whereas, two SMs (K8T and N272S) in the PA4156 gene may affect anaerobic twitching capabilities. The SMs in PA2125 (E395D and H396D) and PA1997 (E11K) identified in AUST-02 strain only appear to be associated with anaerobic swimming. Finally, the indel within the *fimV* gene, demonstrated correlation with loss of motility and adhesion under each different atmospheric condition. This correlation was strongest for swarm and twitching under microaerophilic conditions, as predicted by GWAS, and anaerobic adhesion. Interestingly, this mutation did not appear to functionally affect swimming motility.

Table 6.2 Informative variants and recognised genes which have demonstrated an association with defined phenotypic characteristics following GWAS analysis.

Phenotype	Identified Gene	Variant	Effect of mutation *	Statistical Significance (*P*-value)

Swim	O_2 & MA	*aprE*	upstream modifier	NSM	4.4×10^{-7}
		pscK	Cys152del	NSM	4.4×10^{-7}
		PA1874	Ser150_Gly151dup	NSM	4.4×10^{-7}
Swarm	O_2	*flgK*	Asn372Ser	Unknown	2.2×10^{-2}
	MA	PA1908	Gly91Ala	SM	2.5×10^{-10}
		PA4881	Ile52Val	SM	1.5×10^{-8}
		clpB	Val846Leu	SM	2.6×10^{-8}
		fimV	Gly682_Asp683insGlyAspLeuGlySer	NSM	7.3×10^{-6}
	AnO_2	PA1997	Gly215Asp	SM	2.0×10^{-8}
		hpaA	Ile477Val	SM	2.0×10^{-8}
		PA0209	Ala229Val	SM	5.0×10^{-8}
		xcpQ	Glu417_Ser418delinsAla	Unknown	5.0×10^{-6}
		fimV	Gly682_Asp683insGlyAspLeuGlySer	NSM	2.0×10^{-4}
Twitch	O_2	PA4781	Leu158Phe	SM	1.6×10^{-8}
		PA4782	Ala6Val	SM	1.6×10^{-8}
		PA5484	Glu54Lys	SM	1.6×10^{-8}
		PA5492	Val191Ile	SM	1.6×10^{-8}
		PA0683	E240G	SM	1.6×10^{-8}
		xcpQ	Glu417_Ser418delinsAla	Unknown	7.1×10^{-6}
		pilQ	Gln628FS	Unknown	8.0×10^{-6}
	MA	*fimV*	Gly682_Asp683insGlyAspLeuGlySer	NSM	9.0×10^{-7}
Adhesion	O_2	PA2069	Ala480Glu	SM	7.0×10^{-7}
		PA3292	Ser202Pro	SM	7.0×10^{-7}
		gcvP1	Ser98Pro	SM	7.0×10^{-7}
		opr86	Arg668FS	Unknown	2.6×10^{-5}
		opr86	Thr673_Met675del	Unknown	2.6×10^{-5}
	MA	PA4156	Gly471FS	NSM	1.4×10^{-6}
		opr86	Arg668FS	Unknown	4.0×10^{-6}
		opr86	Thr673_Met675del	Unknown	7.7×10^{-6}
		xcpQ	Glu417_Ser418delinsAla	Unknown	7.0×10^{-5}
		PA0144	Val191Ile	Unknown	9.0×10^{-6}
	AnO_2	PA2125	Lys67Thr	SM	1.8×10^{-6}
		pilQ	Gln628FS	Unknown	1.0×10^{-4}

* As predicted using PROVEAN software.

NSM, nonsynonymous mutation; SM, synonymous mutation

Table 6.3 Synonymous and non-synonymous mutations identified within the genes determined to be significant following GWAS analysis and analysed using gene mapping and assembly.

Origin	CF STRAIN – AUST-02													CF STRAIN – AUST-06						ENVIRONMENTAL STRAINS											Amino Acid Variation (compared to PAO1 *)
Isolate Number	A2-04	A2-06	A2-07	A2-08	A2-11	A2-13	A2-18	A2-19	A2-29	A2-41	A2-43	AUS727	AUS717	AUS883	AUS77	AUS884	AUS885	AUS396	AUS886	AUS222	AUS227	AUS276	AUS306	AUS343	AUS438	AUS449	AUS465	AUS502	AUS503	AUS504	
MLST	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	Novel ST	ST-801	ST-801	ST-801	ST-801	ST-801	ST-209	ST-216	ST-9	ST-381	ST-266	ST-155	ST-930	ST-147	ST-179	ST-810	ST-389	
																															82% NI
aprE																															**USM**
																															W30*
																															E75V
																															A168S
pscK																															RRL161_163R
																															152delC
PA1874																															10-93% NI
flgK																															86-87% NI
PA1908																															29-50% NI
																															S70I
																															G91A
PA4881																															66-97% NI
																															I52V
clpB																															85% NI
																															V846L
fimV																															54-83% NI
																															582_585delKLM
																															GDLGSins683
PA1997																															E11K
																															G135D
																															G215D
																															L290F
h*paA*																															**I477V**
PA0209																															41-56% NI
																															A229V
																															A275V
xcpQ																															92-97% NI
PA4781																															66% NI
																															P65L
																															L158F
																															G339D
PA4782																															**A6V**
PA5484																															97% NI
																															E54K
PA5492																															**V191I**
PA0683																															0-81% NI
																															D83G
																															E240G

Table 6.3, continued Synonymous and non-synonymous mutations identified within the genes determined to be significant following GWAS analysis and analysed using gene mapping and assembly.

Origin	CF STRAIN – AUST-02													CF STRAIN - AUST-06						ENVIRONMENTAL STRAINS										Amino Acid Variation (compared to PAO1 *)
Isolate Number	A2-04	A2-06	A2-07	A2-08	A2-11	A2-13	A2-18	A2-19	A2-29	A2-41	A2-43	AUS727	AUS717	AUS883	AUS77	AUS884	AUS885	AUS396	AUS886	AUS222	AUS227	AUS276	AUS306	AUS343	AUS438	AUS449	AUS465	AUS502	AUS503	AUS504	
MLST	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	Novel ST	ST-801	ST-801	ST-801	ST-801	ST-801	ST-209	ST-216	ST-9	ST-381	ST-266	ST-155	ST-930	ST-147	ST-179	ST-810	ST-389	
pilQ																															90-97% NI
																															A106T
PA2069																															G20D
																															R310H
																															A480E
PA3292																															85-97% NI
																															Y35S
																															G56D
																															Y126C
																															V135E
																															G153E
																															S202P
																															S98P
																															A157G
																															H210Y
gcvP1																															I524N
																															A581V
																															R626C
																															R793L
																															R668FS
																															T673_M673del
opr86																															N669D
																															P670G
																															D671N
																															T673N
																															PMK674_676Q
																															N677I
																															Q678V
																															V715F
PA4156																															0% NI
																															G471FS
																															1 BP del (1413delG)
																															K8T
																															N272S
																															R317H
																															D436N
																															T459I

Table 6.3, continued Synonymous and non-synonymous mutations identified within the genes determined to be significant following GWAS analysis and analysed using gene mapping and assembly.

Origin		CF STRAIN - AUST-02													CF STRAIN - AUST-06						ENVIRONMENTAL STRAINS										Amino Acid Variation (compared to PAO1 *)
Isolate Number		A2-04	A2-06	A2-07	A2-08	A2-11	A2-13	A2-18	A2-19	A2-29	A2-41	A2-43	AUS727	AUS717	AUS883	AUS77	AUS884	AUS885	AUS396	AUS886	AUS222	AUS227	AUS276	AUS306	AUS343	AUS438	AUS449	AUS465	AUS502	AUS503	AUS504	
MLST		ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	ST-775	Novel ST	ST-801	ST-801	ST-801	ST-801	ST-801	ST-209	ST-216	ST-9	ST-381	ST-266	ST-155	ST-930	ST-147	ST-179	ST-810	ST-389	
PA0144																																95-97% NI
PA2125																																46-97% NI
																																G28A
																																K67T
																																D316E
																																L389P
																																E395D
																																H396R
																																D419E
																																V463G
																																D316E
Swim	O_2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	
	MA	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
	AnO_2	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Swarm	O_2	+	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+	-	-	+	
	MA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+	-	+	+	
	AnO_2	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	+	
Twitch	O_2	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+	
	MA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	
	AnO_2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	+	
Adhesion	O_2	-	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	
	MA	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	
	AnO_2	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	

Abbreviations: NI, Nucleotide Identity; O_2, aerobic; MA, microaerophilic; AnO_2, anaerobic; USM, upstream mutation; IFM, in-frame mutation; FS, frame-shift mutation; dup, duplicate; del, deletion; ins, insertion.

Amino acid variations highlighted in Red were identified by GWAS analysis

* PAO1 genome Accession number: NC_002516

No change in protein (nucleotide sequence identical to PAO1 or synonymous substitution detected)

Synonymous mutation not predicted to affect protein (based on Literature search or PROVEAN)

Non-synonymous mutation predicted to affect protein (based on Literature search or PROVEAN), gene missing or large genetic variation detected

Poor sequence quality, gene variant could not be confirmed

6.5 Discussion

This study used two bioinformatics approaches to identify genetic variants which may infer a phenotypic trait. GWAS analysis was initially used as a comprehensive screening tool to identify genetic variants associated with a phenotype. Highly significant variants identified by GWAS were then assessed further using an in-depth genome mapping and assembly approach. This method focused on genetic determinants identified by GWAS as highly correlated with a particular phenotype along with those previously determined to be involved with bacterial motility and adhesion.

Eleven isolates collected from a local river system and 19 isolates obtained from the sputum of patients with cystic fibrosis, chronically infected with *P. aeruginosa*, were analysed. The environmental isolates represented a range of different genotypes, whereas the clinical isolates belonged to one of two clinically relevant clades, AUST-02 or AUST-06 (Kidd *et al.* 2013; Kidd *et al.* 2012). Phenotypically the environmental and clinical isolates possessed very distinct characteristics. Overwhelmingly, the environmental isolates demonstrated a positive phenotypic profile, compared to the clinical strains which lacked the ability to adhere or be motile. However, within the clinical strains, further distinctions were recognised. AUST-06 displayed a more heterogeneous distribution for motility phenotypes compared to AUST-02 isolates, which were almost universally negative. In contrast, no adherence was observed amongst the AUST-06 isolates compared to a number of AUST-02 isolates which were able to adhere.

GWAS analysis did not determine any variants to be completely correlated with swimming motility, regardless of the atmospheric conditions the tests were conducted under. However, two variants, *aprE* and *pscK*, previously characterised, were associated with swimming for this sample set. Interestingly, previous studies have demonstrated that during swarming activity, rather than swimming, these genes were upregulated (Overhage *et al.* 2008; Yeung *et al.* 2009). Results from this study and the previous analyses suggests that these genes may play a role in both flagella driven motilities and additional investigation is needed to determine the extent to which they are influencing motility.

Using a GWAS approach a number of variants within genes (*flgK*, *fimV*, *xcpQ* and *clpB*) which have previously been linked to bacterial adaptation, were shown to be highly correlated with swarming. Of particular interest from this analysis was *clpB*. Both GWAS and genome mapping and assembly determined that this variant was strongly correlated with loss of function. Zhao and colleagues showed that *clpB Bacillus spp.* mutants show reduced swimming motility and fewer flagella compared to a wild type strain (Zhao *et al.* 2016). It is currently unknown if and how a mutation within *clpB* may affect flagella function and what impact this may have on the swarming ability of *P.*

aeruginosa strains; however, PROVEAN analysis indicated that this SNP in the *clpB* (Val846Leu) gene would not deleteriously affect protein function. The remaining genes identified by GWAS, have been previously associated with the flagellum mediated motility, swarming (*flgK*), the Type II secretion system (*xcpQ*) and twitching motility (*fimV*) (Ball *et al.* 2002; Bitter *et al.* 2007; Dasgupta *et al.* 2003; Homma *et al.* 1990; Leid *et al.* 2009; O'Toole and Kolter 1998; Overhage *et al.* 2007; Semmler *et al.* 2000; Van der Meeren *et al.* 2013; Wehbi *et al.* 2011; Zhang *et al.* 2007). While no direct association with swarming was confirmed following assessment of the *flgK* and *xcpQ*, gene sequences, the results from this study suggests that these variants contribute to the loss of swarming motility. Likewise, both approaches determined that an indel within the *fimV* gene was highly correlated with swarm motility, particularly under reduced oxygenated environments. Future mechanistic studies aimed at assessing the role of these genetic determinants in swarming motility are now indicated.

Of note were the results from both analyses when assessing anaerobic twitching. Phenotypically, only three isolates, two environmental strains and one AUST-06, demonstrated a positive twitching phenotype under anaerobic conditions. GWAS was unsuccessful at identifying any specific genetic variants. These results suggest that bacterial respiration may influence impaired twitch motility within this isolate set. Not only has it been demonstrated that *P. aeruginosa* grows at a slower rate in anaerobic conditions, but the expression of many phenotypic traits and genetic pathways are also subdued (Alvarez-Ortega and Harwood 2007; Hogardt and Heesemann 2013; Lee *et al.* 2011; O'May *et al.* 2006; Schobert and Jahn 2010; Waite and Curtis 2009; Wu *et al.* 2005; Yoon *et al.* 2002).

As described in Chapter 5, the flow cell apparatus is superior in determining *in vitro* biofilm development when compared to the microtitre plate based assay. Ideally, the GWAS comparison undertaken in this study would have been based on the flow cell-derived biofilm parameters (as described in Chapter 5) in conjunction with the motility capabilities, rather than the microtitre assay results which are presented here. Due to limitations with isolate selection and availability of sequencing, only a proportion of these isolates underwent both WGS and biofilm flow cell analysis, therefore the microtitre assay was used as a substitute to determine adhesion. Regardless, GWAS successfully identified a range of variants associated with bacterial adherence, as determined by the microtitre plate-based assay, including genes which have been previously associated with bacterial communication (PA0144), iron acquisition (PA4156) and fimbriae function. (PA2125), all of which are essential for the development of bacterial communities (Banin *et al.* 2005; Elias *et al.* 2011; Gilbert *et al.* 2009; Tashiro *et al.* 2008; Vallet-Gely *et al.* 2007). Of particular note was *opr86*, which has previously been associated with bacterial survival and is thought to play an important role in

biofilm formation. Tashiro and colleagues have demonstrated that antibodies against this gene impair biofilm formation (Tashiro *et al.* 2008). The current analysis confirms the importance of this genetic determinant in surface attachment, which is crucial for biofilm formation and development. A six bp *opr86* deletion was observed in the AUST-02 strains, which collectively demonstrated poor adherence to the abiotic surface. This work not only described *opr86* as important for bacterial adhesion, but closer inspection of the SMs identified showed that they correlated with a negative phenotype for swimming anaerobically. Data generated here now provide a platform for further analysis into the genetic mechanisms responsible for adhesion and biofilm formation.

The bioinformatic analyses presented in this study showed synergy among several variants across the different phenotypic characteristics and varied atmospheric conditions. These data suggest that such traits are interlinked and controlled through a range of common genes. The greatest synergy was the swim motility phenotype, with five variants identified under aerobic conditions and microaerophilic conditions (PA3535, PA3536, PA3641, *algE* [Arg10Trp and Gly79Ala]) and four of the five indels also identified across both atmospheres (PA1536, PA1874, *aprE*, *pscK*). Despite being essential for type IV pili biosynthesis and twitching motility (Semmler *et al.* 2000; Wehbi *et al.* 2011), in this isolate set, a significant indel within *fimV* was predicted to affect swarming, twitching and adhesion capabilities.

Another frequently identified variant from GWAS analysis was observed within the *xcpQ* gene; however as the majority of these isolates displayed large genetic variations this particular variant could not be identified. Nevertheless, this genetic variation was associated with twitch motility, swarm motility and adhesion under reduced oxygen conditions. Interestingly, while a direct association with motility or biofilm formation has yet to be confirmed, a study has demonstrated that *xcpQ* mutants retain the ability to develop biofilms (Ball *et al.* 2002; Bitter *et al.* 2007; Overhage *et al.* 2007; Van der Meeren *et al.* 2013). The clinical strains studied here all have low nucleotide identity with the reference strain and displayed impaired adhesion to an abiotic surface. Previous work presented in Chapters 4 and 5 demonstrated that static biofilm formation, focusing on initial adherence, is not predictive of bacterial biofilm formation in a flow cell experimental apparatus. Therefore, results generated from microtitre plate based assay which report that mutations within the *xcpQ* have no effect on phenotype warrant further investigation (Overhage *et al.* 2007). Furthermore, while the role of *xcpQ* in biofilm development remains uncertain, this gene has also been associated with a type II secretion system (Ball *et al.* 2002) that enables excretion of intracellular proteins into the extracellular space. Bacterial toxins associated with the breakdown of tissue and the establishment of infections can be released from the cells in this manner.

An indel in the *pilQ* gene was identified by GWAS during twitching and initial biofilm development under both aerobic and anaerobic conditions. A number of studies have demonstrated a strong association with mutations within this gene to the loss of surface fimbriae and twitching motility (Alm and Mattick 1997; Chang *et al.* 2007; Koo *et al.* 2013). The GWAS performed here identified a significant frameshift mutation in *pilQ* which correlated with anaerobic adhesion; however, the low level of amino acid identity together with several strains lacking this gene entirely may mean that this locus has been mutationally inactivated though multiple mechanisms. When this gene was analysed more closely, this study observed the AUST-02 and the environmental strains demonstrated a high level of sequence variance when compared to the reference strain, yet this did not match the phenotypic capabilities. Overall, the AUST-02 strains demonstrated poor twitching and attachment capabilities, in contrast with the environmental strains that showed enhance traits. All AUST-06 isolates possessed a non-synonymous mutation not predicted to affect protein function; however, like AUST-02 these isolates were non-motile and non-adherent. These results suggest *pilQ* is not solely responsible for this phenotype and that other mechanisms must play a role in bacterial twitching and good adherence. GWAS analysis has shown high levels of synergy across the atmospheric conditions and between the phenotypic traits, highlighting the limitations of using a single approach to assess the impact of genetic variants on phenotypic traits. Rather, a multifaceted approach combining phenotypic, genomic, expression studies and knockout strains is needed to determine if any correlation can be identified.

Results presented in this study need to be interpreted with caution as isolate selection may have influenced these analyses. Two-thirds of the isolates included in this data set were clinical strains, representing two predominant sequence types. In contrast, the environmental strains represented 11 STs and each strain had a unique sequence type. As demonstrated the GWAS, many of the genetic variants identified as significant for a particular trait have been previously associated with antibiotic susceptibility rather than motility and adhesion and the results presented here may be influenced by the number of highly antibiotic resistant clinical strains. Therefore, if this approach is to be used to identify targets for further genomic analysis a larger isolate cohort should be used, including a broader range of isolates with varied genotypes, sourced from a range of different clinical and environmental settings (e.g. non-CF) and possessing a range of different phenotypic traits (e.g. antimicrobial resistance). Unlike the CF strains which have adapted within a unique environment having prolong exposure to high doses of antibiotics and a range of host defenses, isolates from acute infections are not likely to have undergone bacterial adaptation and are less likely to have developed acquired resistance. Additionally, the phenotypic heterogeneity and relatively small population within the

isolate categories (environmental, AUST-02 and AUST-06) reduce the power of the GWAS analysis. Despite technical replicates being performed on each isolate, repeat phenotypic testing is required to confirm any unexpected results. Finally, a large number of hypothetical proteins were also identified following GWAS analysis. As these genes have not been previously described further analysis using mapping and assembly techniques were not undertaken. Nevertheless, these variants may also play an important role in the expression of phenotypic characteristics. As such, these variants may provide a number of potential candidates for a more targeted approach to assess genes associated with motility and adhesion in future studies. While this study was unable to identify a single genetic variant that was associated with a discrete phenotypic trait, the methodology presented here could be adapted and used to identify the genetic underpinnings of particular bacterial phenotypes and clinical outcomes. As whole genome sequencing and bioinformatic analysis is increasing and the technology will be important in the development of diagnostic markers able to assist in rapid diagnosis. However, to better understand the mechanisms associated with *P. aeruginosa* motility and adhesion a larger sample size is required for bioinformatic analysis and to be able to determine clinical impact of these traits on the health outcomes for patients, corresponding clinical characteristics also need to be assessed.

This study identified a number of genetic variants, which likely influence the phenotypic characteristics associated with development of chronic infections. It is anticipated that this preliminary analysis will form the basis of ongoing work; including additional GWAS and a targeted mutation approach to functionally assess the most significantly associated genetic variants. Finally, no single genetic mutation accounted for a discrete phenotype, which is likely to reflect the diversity and evolutionary history of the isolates studied. These analyses suggest parallel evolution of the AUST-02 and AUST-06 clades has occurred as these groups have evolved similar phenotypes through different genetic alterations. Through the inclusion of additional isolates and expansion of genetic analysis to include knockout strains, it is hoped that this work will further contribute to the existing knowledge of bacterial adaptation.

Chapter 7: Overall Discussion and Thesis Conclusions

7.1 Hypothesis and Aims

To date the prevalent *P. aeruginosa* shared strains commonly isolated from CF patients residing in Queensland have not been isolated from other clinical or environmental settings. It is hypothesised

that these strains possess specific mechanisms which enable persistence and adaptation to the CF airways, different to other commonly isolated strains. Therefore, using the phenotypic and genomic analysis described in this PhD, comparison studies to elucidate these differences were undertaken. Motility, adhesion and biofilm development were chosen as these traits have been associated with antibiotic resistance, adaptation, virulence and infection persistence, all mechanisms relevant and important for infection development within the CF airways. The specific methodologies used throughout this thesis are commonly used and cited techniques to assess these traits.

7.2 Key Findings of thesis

7.2.1 Chapter 2: Prevalence of *Pseudomonas aeruginosa* in an adult CF centre

The data presented here is the first time the microbial pathogens present in the sputum of adult patients attending The Prince Charles Hospital have ever been collated and reported.

The key findings from this chapter include demonstrating that:

1. The prevalence of significant respiratory infections, including *P. aeruginosa*, has decreased over time.
2. There has been an improvement in the lung function and nutritional status of young patients at the time of transition into the adult centre.
3. The current protocol used to define chronic respiratory infections caused by *P. aeruginosa* (Lee *et al.* 2003) was unsuitable when applied to a contemporary adult patient population.

The most significant contribution to the current knowledge of CF microbiology was:

Modifications to the definition describing chronic *P. aeruginosa* to better represent patient sampling within a contemporary adult population.

7.2.1.1 Limitations

As outlined in Chapter 2 the major limitation of this study centred on the sampling and culture techniques utilised to generate these data. More specifically, the use of culture dependent techniques may have under estimated the airway microbial diversity within this patient population.

This work would also have benefited from additional data pertaining to childhood eradication therapy following *P. aeruginosa* acquisition. Indeed, inclusion of details relating to *P. aeruginosa* acquisition, treatment regimens and outcomes would have further aided in the categorisation of patients. Comparisons between patients based on treatment regimens would have been used to determine if successful eradication delays the onset of chronic infection and if subsequently these patients were at a higher risk of respiratory infections caused by other common and emerging CF airway pathogens.

7.2.1.2 Implications of Findings and Future Work

As the number of patients reaching adulthood continues to grow, a significant challenge moving forward will be caring for patients, who are healthier than previous generations, within clinics with limited resources and finite funding. Therefore, approaches to help manage this increase including strategies to monitor and quantify changes to patient health are of utmost importance. Patient databases or data registries, collecting demographic and anthropometric measurements from patients can be used to monitor trends within a clinic and to assess patient health and outcomes against the

wider CF community. Therefore, clinical data registries play an important role in observing and reporting on the health of patients and can inform treatment and clinical practice.

The development of an extensive microbiology database described in this Chapter can be linked with the local electronic medical records and national data registries (metadata). This has already been undertaken by our group to support the investigation of emerging CF pathogens such as NTM infection (Sherrard *et al.* 2017) and will be extended to collate data specifically used to screen, monitor and diagnose these 'emerging' health problems in the growing adult CF population. For example clinical data pertaining to increasing antibiotic exposure, such as allergies and toxicity, diseases related to lifestyle such as obesity and heart disease, the emergence of antibiotic-resistant airway pathogens, and others such as malignancy, bone disease and mental health illnesses will be included.

Future studies planned to complement the findings presented in this chapter will include:

i) The continuation of monitoring microbial prevalence and rates of chronic *P. aeruginosa*, within the patient population at TPCH.

ii) Assessment of the effectiveness of childhood eradication therapies to determine how successful they are in predicting bacterial infection in adulthood.

iii) Evaluation of the effectiveness of eradication programs in adults who are free of chronic *P. aeruginosa infection.*

iv) Establishment of a database to record clinical information regarding the onset of 'emerging clinical complications' to improve the management of an ageing CF population.

7.2.2 Chapter 3: Longitudinal analysis of *Pseudomonas aeruginosa* genotypes

Utilising two cross sectional collected isolate cohorts, this study assessed the prevalence, diversity and clinical impact of *P. aeruginosa* isolates collected from adults attending a single CF centre and determined that:

1. Strain diversity and prevalence rates can fluctuate over time.
2. Clinical outcome appears to be strain specific

The most significant contribution to the current knowledge of CF microbiology was:

In the absence of enhanced infection control practices, not all shared strain infections increase, indicating that mechanisms specific to the individual strains may be influencing transmissibility and infection.

7.2.2.1 Limitations

As described in Chapter 3, the key limitation of this work focused on the small number of isolates available for genotyping; therefore, it is feasible that *P. aeruginosa* genotypes in some patients have been underrepresented. Nevertheless, within-patient genotyping results generated in this study were similar to earlier work and indicate that the vast majority of patients are infected with a single *P. aeruginosa* strain (Kidd *et al.* 2013).

The typing methodology utilised in this study can accurately discriminate the predominant Australian CF shared strains (i.e. AUST-01, AUST-02). However, compared to other molecular typing methods, the SNP typing strategy used here has a reduced capacity for discriminating between some less prevalent and unique *P. aeruginosa* strains (Syrmis *et al.* 2014). As such, the current analysis may have overlooked the emergence of some novel shared strain infections. Nevertheless, a thorough examination of this dataset looking for emergent strains revealed no cases.

Unlike many previous studies describing the diversity and prevalence of *P. aeruginosa* strains within defined CF populations, this work did not include patient data pertaining to treatment regimens and co-pathogens. Therefore, this analysis was unable to comment on the impact of shared strain acquisition with respect to these variables within this cohort.

Finally, while this study was designed to capture all incidence cases of shared strain infection and corresponding clinical data; it was not designed to capture these same data for the acquisition of a new non-shared strain infection. Therefore, accurate comparison of the clinical impact and risk factors associated with shared and non-shared strain infection could not be made.

7.2.2.2 Implications of Findings and Future Work

This study reported several important findings following the genotyping of *P. aeruginosa* isolates within TPCH ACFC patient population, including: i) the majority of patients harbour shared strain infection rather than a minor shared or unique strain infections, ii) over recent years the prevalence of the AUST-06 shared strain substantially increased, iii) the prevalence of the AUST-02 shared strains appears to be decreasing and iv) the proportion of patients harbouring the AUST-01 shared strains has remained stable over time. Furthermore, this analysis demonstrated an association with an increased risk of death or transplantation for AUST-02 infection and a trend towards increased hospitalisations and lower lung function for AUST-06.

The results presented here are independent of any intervention such as patient segregation based on *P. aeruginosa* genotype. However, following the commissioning of a new inpatient facility at the ACFC, TPCH in 2014, patients are now managed in single room accommodation which has the potential for reducing the risk of cross-infection. This change in practice coupled with longstanding strict infection control practices in the outpatient clinic, has led to a prospective study to determine the impact of the changes in care procedures leveraging from the results presented in Chapter 3. This study will determine the impact on rates of shared strain infection and their impact on clinical outcomes.

Future studies planned to complement the findings presented in this chapter will include:

i) To conduct a prospective epidemiological analysis examining strain diversity at TPCH following the implementation of complete patient segregation. This study is funded and commenced in 2016.

ii) If subsequent *P. aeruginosa* infection ensues following lung transplantation, conduct genotyping of these isolates to determine if persistence in the upper respiratory tract has occurred.

iii) Undertake additional analysis on AUST-02 and AUST-06 strain, focusing on virulence and antibiotic resistance, to better understand why infection with these strains result in such different clinical courses.

7.2.3 Chapter 4: Phenotypic characteristics of *Pseudomonas aeruginosa*

Bacterial motility and adherence play an important role in the colonisation and the establishment of infection. Results from this study demonstrated that *P. aeruginosa* isolates obtained from different clinical and environmental niches display very distinct phenotypic characteristics.

The key finding from the phenotypic analysis undertaken here showed that:

1. Isolates which demonstrate enhance motility also display strong adhesion.
2. Isolates obtained from the environment displayed enhanced motility and adhesion traits compared to all other isolates.
3. The majority of isolates obtained from acute clinical infections (non-CF and animal sources) demonstrated the ability to adhere and be motile.

The most significant contribution to the current knowledge of CF microbiology was:

Isolates from chronically infected CF patients, in particular the shared strain, AUST-02, showed reduced motility and adhesion, suggesting high levels of adaptation to the airways.

7.2.3.1 Limitations

As described in Chapter 4 the key limitations of this work were the overrepresentation of the AUST-02 strains, comprising one in three isolates studied here, and omission of AUST-02 sub-type information. Due to the limited diversity of sequence types included in this study, it is difficult to conclusively infer an association between genotype and phenotype. Therefore, with the inclusion of a greater variety of strains from people with CF, this study would have increased the power to confirm the phenotypic behaviours observed between isolates of different genetic backgrounds. The data selection and omission of more in-depth genotyping results may have led to the underestimation of the diversity of motility and adhesion within this isolate cohort.

This study would also have benefited greatly from the inclusion of fitness studies. Currently it is unknown if the display of reduced phenotypic characteristics have been misinterpreted and rather these results are due to slow bacterial generation times. Therefore, determining the growth rates of bacterial isolates would have future enhanced the significance of the data presented in this chapter.

7.2.3.2 Implications of Findings and Future Work

The work presented in Chapter 4 was the first analysis undertaken on a cohort of locally collected isolates from a range of clinical and environmental niches to describe phenotypic characteristics. This work demonstrated reduced bacterial motility and abiotic adherence in the AUST-02 shared CF strain, whereas non-shared CF strains do not display the same degree of suppression. A study to more closely

assess the strains, functional cell surface organelles and the impact on patient health is planned. This will be a longitudinal study following a small number of patients from initial infection onwards. Two patient cohorts will be targeted, those who acquire AUST-02 and those who acquire a non-shared strain. It is hypothesised that despite being a new infection the AUST-02 strain will not possess functional cell surface organelles needed for motility and adherence, whereas, longitudinal monitoring of the non-shared strain, will report a gradual loss of function over time. These results will be matched with relevant clinical data to assess if a decline in bacterial phenotype corresponds with a decline in patient health. By monitoring of the loss of phenotypes, this study may help inform disease progression and along with other clinical and microbiological markers may identify strains which could potentially result in adverse clinical outcomes.

As discussed in Chapter 3, there has been a rapid increase in the prevalence of the AUST-06 shared strain in recent years at TPCH. To better understand mechanisms, which may be influencing transmission and infection of this strain, phenotypic characteristics will be analysed on a larger cohort of isolates, including strains from early and chronic infection of AUST-06.

Future studies planned to complement the findings presented in this chapter will include:

i) To conduct a longitudinal study, utilising initial infecting stains, to more accurately determine if infection duration is:
    a. Associated with changes to bacterial phenotype and
    b. If change to bacterial phenotype corresponds to a decline in patient health.

ii) Determine, using a subset of isolates with varied phenotypic characteristics, the presence or absence of the cell surface organelles using scanning electron microscopy and staining.

iii) Assess the phenotypic characteristics of a larger cohort of AUST-06 strains to:
    a. Compare characteristics to AUST-02 strains
    b. Determine if phenotypic characteristics may be responsible for increased transmissibility.

7.2.4 Chapter 5: Biofilm development of *Pseudomonas aeruginosa*

The continuous flow cell model was used here to determine if there was a correlation between biofilm development, bacterial adhesion as determined in the static biofilm model and motility.

The key finding from this analysis demonstrated that:

1. Clinical strains of *P. aeruginosa* formed larger biofilms consisting of greater biomass than those formed from isolates obtained from the environment.
2. No change in the ability to form biofilms was noted from longitudinally collected isolates from chronically infected CF patients.

The most significant contribution to the current knowledge of CF microbiology from Chapter 5 was: Adhesion capability as determined by the microtitre assay did not infer biofilm development within the flow cell model.

7.2.4.1 Limitations

The limitations of this study are described in detail in Chapter 5, however these include, a potential selection bias (all the isolates from patients were AUST-02 strains, whereas, the environmental isolates were more heterogeneous, including unrelated strains) and the omission of early or initial infecting strains for longitudinal analysis. Using this isolate selection, comprising mostly of AUST-02 strains from chronic infections it is difficult to determine if genotype or degree or bacterial adaptation is responsible for biofilm formation.

A number of technical challenges were encountered whilst developing the protocols and setting up these experiments. Of significant note was the introduction of fluorescent stain by injection into the chamber. The use of this method to aid visualisation may have disturbed the delicate bacterial biofilm structure and as this was manually performed, leading to reduced reproducibility between biofilm experiments. The alternative approach, green fluorescent protein (GFP) was not utilised for this set of experiments.

7.2.4.2 Implications of Findings and Future Work

A key finding of this investigation was that the static microtitre plate biofilm assay designed to assess bacterial adhesion is not a reliable predictor of *P. aeruginosa* biofilm forming capacity in the continuous flow-cell model. Therefore, the use of static models to infer biofilm capacity is limited. Furthermore, continuous flow-cell models which support the study of dispersed cells may provide understanding of adaptation mechanisms and allow comparison with their parental strain.

Complementary studies to assess the biofilm capacity of a number of isolates and conducting comparisons with this original work have been planned. Isolates to be assessed in this follow up study include: previously identified significant shared strains within the TPCH cohort, including AUST-06 (recent increase in prevalence as outline in Chapter 3) and the resistant AUST-02 sub-type, M3L7 (Tai *et al.* 2015), as well as a range of non-CF and animal isolates as detailed in Chapter 4. These additional analyses will determine if AUST-02 shared strain infection is unique in its biofilm producing phenotype compared with other CF and non-CF strains. In addition, analysis of isogenic isolates (including AUST-02 and AUST-06) obtained periodically from early to chronic infection will be performed to determine if biofilm producing capacity evolves in conjunction with the evolution of infection.

Finally, studies to assess how strains behave under exposure to environmental and clinical stressors will be conducted. Firstly, two murine models, a cutaneous abscess model and a chronic airway infection model, will be used to assess the growth and infection development of shared strains and non-CF isolates (Pletzer *et al.* 2017). It is hypothesised that AUST-02, which displays a high level of niche adaptation to the CF airways, will behave in a similar manner to all non-CF isolates under cutaneous infection conditions and will respond to eradication attempts, whereas, in the airway model this strain will display traits associated with enhanced adaptation (Facchini *et al.* 2014). Secondly, in collaboration with environmental scientists located in South Eastern Queensland, *P. aeruginosa* strains, including the AUST-02 will be inoculated into diffusion chambers and placed into a local waterway (Sidhu and Toze 2012). These experiments will assess bacterial decay, phenotypic, genomic and protein expression during and following a period of re-introduction into the natural environment. Antimicrobial susceptibility testing will be performed on all isolates to determine if a dispersal event results in the release of susceptible organisms back into the local environment.

Future studies planned to complement the findings presented in this chapter will include:

i) A range of clinically significant shared strains, non-CF and animal isolates will be assessed in the biofilm model and compared to results generated from AUST-02 and environmental strains.

ii) Determine if isolates released during biofilm dispersal events differ from the original parental strains by:

   a. Undertaking phenotypic characterisation of daughter isolates and
   b. Performing antimicrobial susceptibility testing on both parental and daughter isolates.

iii) Using murine models assess the virulence of AUST-02 strains when compared to isolates obtained from acute non-CF infections and environmental settings.

iv) To determine if environmental exposure will affect the phenotypic and genomic expression of isolates sourced from a range of clinical and environmental niches.

7.2.5 Chapter 6: Genomic analysis of *Pseudomonas aeruginosa*

A genome-wide association study (GWAS) was performed on *P. aeruginosa* isolates displaying distinct phenotypic characteristics. Genome mapping and assembly was performed to locate, confirm and functionally characterise the polymorphisms identified by GWAS in each individual isolate. These results were compared and correlated with phenotypic data to determine if any gene variant could predict a specific phenotypic characteristic.

The key finding from this analysis revealed:

1. Regulation of bacterial motility and adhesion is complex and multifactorial.
2. Several novel targets for which future mechanistic analyses can be undertaken.

The most significant contribution to the current knowledge of CF microbiology was:

Despite strong correlations observed in the GWAS analyses, no single genetic variant was able to fully explain the phenotypes observed.

7.2.5.1 Limitations

Given the cost and the complexity of these analyses only a small number of isolates were included in this study. Regardless, this study was able to demonstrate that many of the variants observed in the shared strains were present in a high proportion of the isolates analysed. It is likely that analysis of an expanded number of isolates would further strengthen these findings.

For this initial study, select variants were chosen for additional analysis using the mapping and assembly approach. Firstly, all variants shown to be highly correlated with a phenotype as described by GWAS and phylogenetic analysis were selected. Secondly, based on results from a literature review only those variants currently associated with the aforementioned phenotypes were selected. This approach may have missed variants, in particular hypothetic proteins, with a strong correlation to a phenotype, but as all of the 'strongly correlated variants' as described by GWAS were followed up, this is unlikely.

Five of the selected genes (*flgK*, *xcpQ*, *pilQ*, PA0144, PA1874) contained large genetic variations, for most if not all of the isolates, therefore mapping and assembly could not be undertaken. To assess if these genetic variations may be causing a loss of function, comparative genetic analyses utilising sophisticated bioinformatic software will need to be performed. This specialised analysis was outside the scope of this study, but is planned for future studies.

7.2.5.2 Implications of Findings and Future Work

Using this dual approach these studies were able to identify a number of variant genetic loci which were strongly associated with bacterial motility and adhesion. To confirm their relationship with the relevant phenotype these genes will now undergo site directed mutagenesis with the mutants generated assessed in the relevant motility and/or adhesion assays. In addition, each knockout mutant showing a negative phenotype will undergo complementation with a wild-type copy of the relevant gene to confirm the genetic mechanism.

Following on from the genomic analyses, a subset of isolates with different phenotypic and genomic profiles will undergo proteomic analysis. As well as assessing bacteria during planktonic growth, this approach allows for analysis to be conducted during different stages of biofilm development. This will prove to be helpful in determining the changes in expression associated with acute (planktonic) and chronic (biofilm) infections. Together with the genomic analysis, the proteomics will help to better understand the mechanisms involved during the establishment of an infection and may ultimately provide markers which may indicate if an isolate will respond to eradication therapy.

Future studies planned to complement the findings presented in this chapter will include:

i) Undertake a targeted analysis of the remaining variants and indels as identified by GWAS to assess correlation with specific phenotypes.

ii) Undertake comparative genomic analysis to describe the mechanisms causing large genetic variations within this isolate cohort.

iii) Using site directed mutagenesis, confirm that targeted mutations are associated with a loss of function.

iv) To compare protein expression when strains of *P. aeruginosa* are grown in planktonic and biofilm growth modes to determine how growth mode affects phenotypic capabilities.

7.3 Concluding remarks

Through the use of locally obtained *P. aeruginosa* isolates and the evaluation of a defined adult CF population, this thesis demonstrates high levels of strain diversity and phenotypic heterogeneity is common. Collectively the results presented in this thesis demonstrate a reduction in significant airway pathogens coupled with improved lung health outcomes of patients within a large adult CF cohort. Furthermore, high levels of strain diversity and heterogeneity was observed within the *P. aeruginosa* isolates from this patient cohort. Observations from these studies contradict prior analysis, confirming that cellular mechanisms driving bacterial motility and adhesion are not invariably required for the development of biofilm communities.

Over the last decade much work has been conducted in describing the clinical aspects and microbiology of the patients in attendance at TPCH. Collectively, these studies have highlighted the presence of three dominant shared strains within this clinic. Closer analysis of AUST-02, including the additional evidence presented within this thesis, has demonstrated that this strain possesses numerous traits which lead it to being highly niche adapted to the CF airways. It is hypothesis that these strains have been circulating within the CF population for an extended period, and in turn have evolved to survive under the particular conditions in which they are exposed. They have adapted to avoid host defenses enabling proliferation but have also evolved to down regulate functions which are no longer advantageous to survival. Therefore, it is speculated that the high level of evolutionary changes which have taken place will no longer support survival in an environment outside the airways. Finally, a commonly utilised method to infer bacterial biofilm development was shown not to be consistent with results obtained from a continuous flow-cell model, indicating that caution when interpreting results needs to be taken.

It is envisaged that work described in this thesis will assist in highlighting mechanisms used by *P. aeruginosa* to enhance niche adaptation within the CF airways and ultimately contribute to therapies aimed at eradication.

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Appendix 1 Published Works Incorporated into the Thesis

Ramsay, K.A., H. Sandhu, J.B. Geake, E. Ballard, P. O'Rourke, C.E. Wainwright, D.W. Reid, T.J. Kidd, and S.C. Bell (2017). The changing prevalence of pulmonary infection in adults with cystic fibrosis: A longitudinal analysis. *J Cyst Fibros. 16(1): p. 70-77.*

http://www.sciencedirect.com.ezproxy.library.uq.edu.au/science/article/pii/S1569199316305677

Justification to include the published manuscript into this thesis

Throughout my research career I have been closely involved with a number of studies focusing on the epidemiology and transmissibility of *Pseudomonas aeruginosa* within the Australian cystic fibrosis (CF) population. These studies determined that within this cohort, two predominant shared strains, AUST-01 and AUST-02, were highly prevalent, isolated nationally from 22% and 18% of patients with CF, respectively. Furthermore, a number of strains appeared to have an affinity for a particular region or patient group. Of note, the AUST-02 strain was most commonly detected in Queensland and Western Australia, whereas the AUST-06 strain was almost universally identified from patients residing in South East Queensland (Kidd *et al.* 2013). Complementary to this study, an extensive epidemiological survey was unable to isolate shared strains in any setting (non-CF and animal infections, environmental isolates) other than the CF airway (Kidd *et al.* 2012). Through the use of innovative technology this group determined that patients harbouring *P. aeruginosa* within their airways are capable of coughing viable organisms into their immediate surroundings (Knibbs *et al.* 2014). Collectively these studies suggest that patients are not acquiring shared strains from common environmental sources and furthermore highlight the high level of niche adaptation to the CF airways which these strains display.

Following on from these initial studies, my thesis aimed to understand the longitudinal epidemiology of *P. aeruginosa* infection in CF and to determine traits which may promote niche adaptation among shared CF strains. The overarching focus of my thesis was to thoroughly characterise a defined set of *P. aeruginosa* isolates obtained from a single patient population. By comparing the CF isolates to locally collected environmental and non-CF clinical isolates, I aimed to determine the key differences in phenotypic and genomic mechanisms and how they may influence adaptation.

Results presented in this manuscript therefore comprise the initial study of my PhD. Focusing on CF patients managed within The Prince Charles Hospital (TPCH) in South East Queensland, Australia; this study presents four key findings. Firstly, this study describes the demographic and clinical

characteristics of patients over a 14 year period. Secondly, *P. aeruginosa* was identified as the most dominant pathogen within this patient cohort; however the prevalence of this bacterium and many other significant airway pathogens declined over the study period. Thirdly, due to the nature of patient attendance and data collection, the Leeds criteria (Lee *et al.* 2003) could not be utilised to determine chronic *P. aeruginosa* infection; therefore, following modifications to this definition it was demonstrated that chronic infection remained stable over time. Finally, this work demonstrated that the declining prevalence of *P. aeruginosa* in young patients showed a correlation with lung function improvement.

Utilising isolates obtained from a subset of patients described in this manuscript, Chapter 3 defines the longitudinal diversity of *P. aeruginosa* strains within the TPCH cohort. Additionally, Chapter 3 reports the clinical impact of stable and newly acquired shared strain infection. Following on from these two chapters, which clearly describe the TPCH cohort, Chapters 4, 5 and 6 use a number of phenotypic assays and genomic approaches to define the characteristics and mechanisms which distinguish *P. aeruginosa* strains collected from people with CF (as presented in Chapter 3), with those obtained from a range of clinical and environmental settings (Kidd *et al.* 2012).

Overall, the work presented in this manuscript forms the foundation of this entire PhD thesis and presents data pertinent to patients attending this CF centre within South East Queensland. As such, I believe that the inclusion of this work is valid.

Appendix 2 Additional Published Works Relevant to the Thesis but not Forming Part of it.

Ramsay, K. A., R. E. Stockwell, S. C. Bell and T. J. Kidd (2016). Infection in cystic fibrosis: impact of the environment and climate. *Expert Rev Respir Med 10(5): 505-519.*

http://www.tandfonline.com.ezproxy.library.uq.edu.au/doi/abs/10.1586/17476348.2016.1162715

Appendix 3 Appendix to Chapter 4

The following appendix constitutes data mentioned in Chapter 4: Phenotypic characteristics of *Pseudomonas aeruginosa*.

Appendix 3.1 *P. aeruginosa* isolate selection for motility and adhesion assays obtained from environmental, animal, non-CF human and CF sources, n = 167.

Appendix 3.2 Representative images of different *P. aeruginosa* colonial morphotypes, a) non-mucoid, non-pigmented, b) mucoid, non-pigmented, c) pyocyanin production, and d) pyomelanin production.

Appendix 3.3 Representative images of positive and negative *P. aeruginosa* motilities a) swim, b) swarm c) twitch (extended methodology).

Appendix 3.4 A representative image of biofilm formation of *P. aeruginosa* using the microtitre plate-based assay.

Appendix 3.5 Limits of agreements determining the reproducibility of a) swim, b) swarm and c) twitch motility assays for *P. aeruginosa* isolates tested under aerobic, microaerophilic and anaerobic conditions.

Appendix 3.6 Comparison of motility and adhesion properties tested under aerobic and anaerobic atmospheric conditions for *P. aeruginosa* isolates obtained from patients with CF, non-CF human infections, animal infections, and environmental settings after 24 hours incubation.

Appendix 3.7 Comparison of two methodologies used to determine twitch motility of *P. aeruginosa* isolates.

Appendix 3.8 Pearson correlation coefficient results of log transformed data comparing swim, swarm and twitch results following 24hours incubation in microaerophilic and anaerobic conditions for *P. aeruginosa* isolates.

Appendix 3.9 Limits of agreements determining the reproducibility of the microtitre plate-based assay for *P. aeruginosa* isolates tested under a) aerobic, b) microaerophilic and c) anaerobic conditions.

Appendix 3.10 Spearman correlation coefficient results of log transformed data comparing swim (mm^2), swarm (mm^2) and twitch (mm^2) with adhesion (OD_{595nm}) results after 24-hours incubation under each aerobic, microaerophilic and anaerobic atmospheric conditions for *P. aeruginosa* isolates.

Appendix 3.1 *P. aeruginosa* isolate selection for motility and adhesion assays obtained from environmental, animal, non-CF human and CF sources, n = 167.

Isolate Selection

The isolate selection for this study was based on results from epidemiological studies reporting the strain diversity of *P. aeruginosa* isolated from the clinical and environmental samples collected within the region of South Eastern Queensland, Australia (Kidd ERJ 2013, Kidd PloS One 2012). The strain types assessed here present the most commonly identified strain types, unique strains and strains identified in each of the different environmental niches.

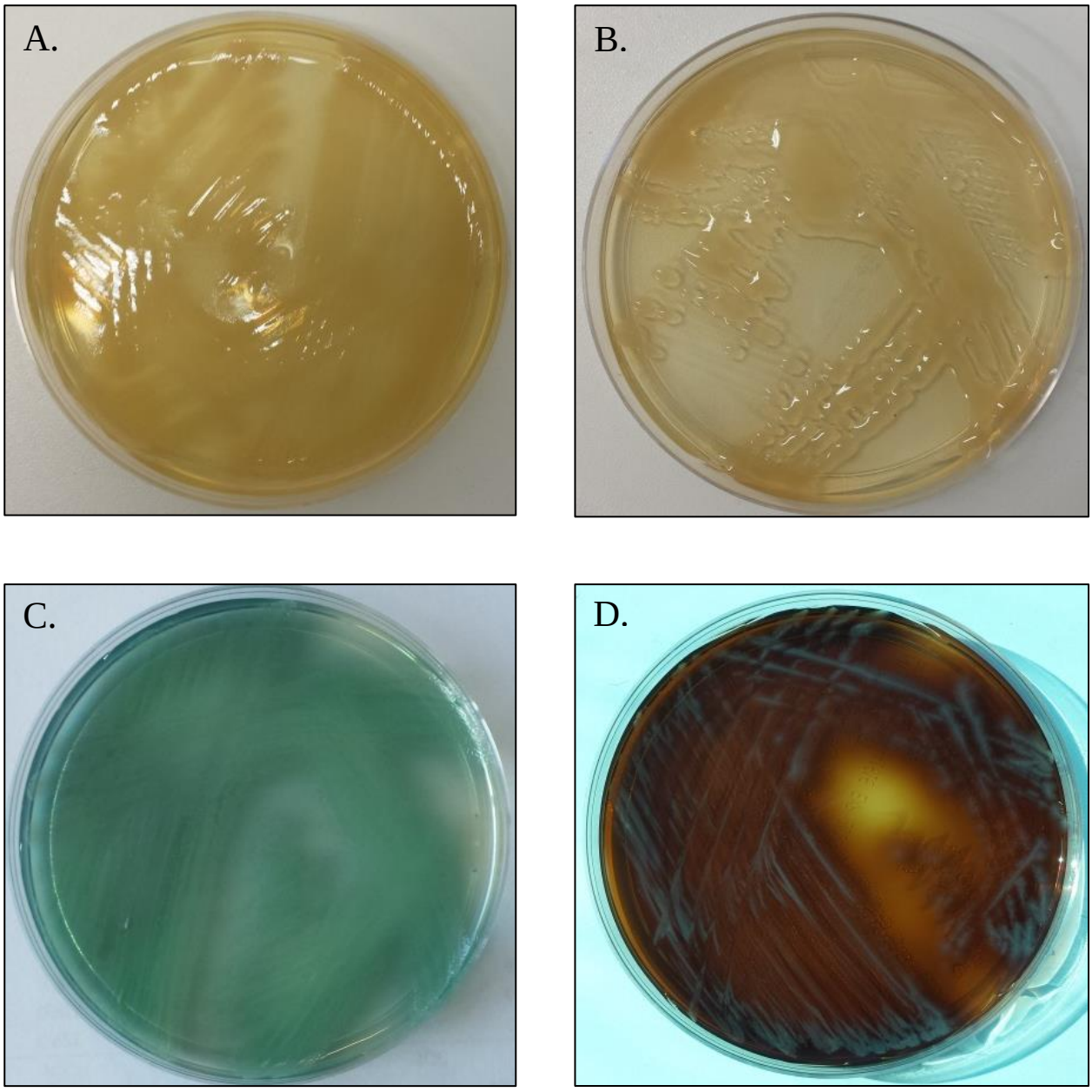
Number	Source	Sample Type	Additional sample & genotype information	Sequence Type
1	Environment	Swab; Air/Water Interface	Brisbane River; Rural	3
2	Environment	Water	Brisbane River; Urban	9
3	Environment	Swab; Household sink drain	Home Study	27
4	Environment	Water	Brisbane River; Urban	114
5	Environment	Swab; Air/Water Interface	Brisbane River; Rural	144
6	Environment	Water	Brisbane River; Industrial	147
7	Environment	Water	Brisbane River; Urban	155
8	Environment	Swab; Air/Water Interface	Logan River; Rural	179
9	Environment	Water	Brisbane River; Urban	205
10	Environment	Swab; Air/Water Interface	Brisbane River; Rural	209
11	Environment	Water	Brisbane River; Urban	216
12	Environment	Swab; Air/Water Interface	Brisbane River; PA14; Urban	253
13	Environment	Water	Brisbane River; Urban	257
14	Environment	Water	Brisbane River; Rural	266
15	Environment	Water	Brisbane River; Urban	270
16	Environment	Water	Brisbane River; Urban	381
17	Environment	Swab; Air/Water Interface	Logan River; Urban	389
18	Environment	Water	Brisbane River; Urban	471
19	Environment	Swab; Air/Water Interface	Brisbane River; Industrial	532
20	Environment	Swab; Air/Water Interface	Brisbane River; Urban	555
21	Environment	Water	Brisbane River; Urban	685
22	Environment	Water	Brisbane River; Industrial	802
23	Environment	Swab; Air/Water Interface	Logan River; Urban	810
24	Environment	Swab; Air/Water Interface	Brisbane River; Rural	838
25	Environment	Swab; Air/Water Interface	Brisbane River; Rural	843
26	Environment	Swab; Air/Water Interface	Brisbane River; Urban	850
27	Environment	Swab; Air/Water Interface	Brisbane River; Urban	855
28	Environment	Swab; Air/Water Interface	Brisbane River; Rural	867
29	Environment	Swab; Air/Water Interface	Brisbane River; Rural	870
30	Environment	Swab; Air/Water Interface	North Pine River; Rural	922
31	Environment	Swab; Air/Water Interface	Logan River; Rural	923
32	Environment	Swab; Air/Water Interface	Brisbane River; Urban	925

33	Environment	Swab; Air/Water Interface	Brisbane River; Urban	927
34	Environment	Water	Brisbane River; Rural	930
35	Animal	Swab; Ear	Canine	155
36	Animal	Milk	Caprine	179
37	Animal	Swab; Clitoris	Equine	242
38	Animal	Respiratory Sample	Canine	244
39	Animal	Swab; Ear	Canine; PA14	253
40	Animal	Swab; Ear	Canine	266
41	Animal	Tracheal Washing	Feline	274
42	Animal	Urine	Feline	381
43	Animal	Swab; Ear	Canine	471
44	Animal	Swab; Ear	Canine	508
45	Animal	Swab; Ear	Canine	789
46	Animal	Swab; Ear	Canine	803
47	Animal	Swab; Uterus	Equine	883
48	Animal	Swab; Ear	Canine	893
49	Non-CF Human	Blood; Peripheral	Clone C	17
50	Non-CF Human	Sputum	Nosocomially Acquired	27
51	Non-CF Human	Swab; Ear		147
52	Non-CF Human	Swab; Ear		155
53	Non-CF Human	Urine; IDC		179
54	Non-CF Human	Sputum	Community Acquired	242
55	Non-CF Human	Urine; Mid-Stream		244
56	Non-CF Human	Blood; Peripheral		245
57	Non-CF Human	Blood; Peripheral	PA14	253
58	Non-CF Human	Urine		266
59	Non-CF Human	Swab; Wound Left Foot		270
60	Non-CF Human	Sputum	Bronchiectasis	274
61	Non-CF Human	Blood; Peripheral		275
62	Non-CF Human	Swab; Cellulitis		381
63	Non-CF Human	Blood; Peripheral		803
64	Non-CF Human	Sputum	Bronchiectasis	807
65	Non-CF Human	Sputum	COAD	821
66	Non-CF Human	Sputum	COAD	845
67	Non-CF Human	Urine; Mid-Stream		860
68	Non-CF Human	Sputum	Bronchiectasis	914
69	CF (Paediatric)	Sputum	Non-Shared CF Strain; Clone C	17
70	CF (Paediatric)	Sputum	Non-Shared CF Strain	27
71	CF (Adult)	Sputum	Shared CF Strain; LES	146
72	CF (Adult)	Sputum	Shared CF Strain; LES	146
73	CF (Adult)	Sputum	Shared CF Strain; LES	146
74	CF (Adult)	Sputum	Shared CF Strain; LES	146
75	CF (Adult)	Sputum	Non-Shared CF Strain	147
76	CF (Paediatric)	Sputum	Non-Shared CF Strain	155
77	CF (Paediatric)	Sputum	Non-Shared CF Strain	179
78	CF (Adult)	Sputum	Non-Shared CF Strain	242

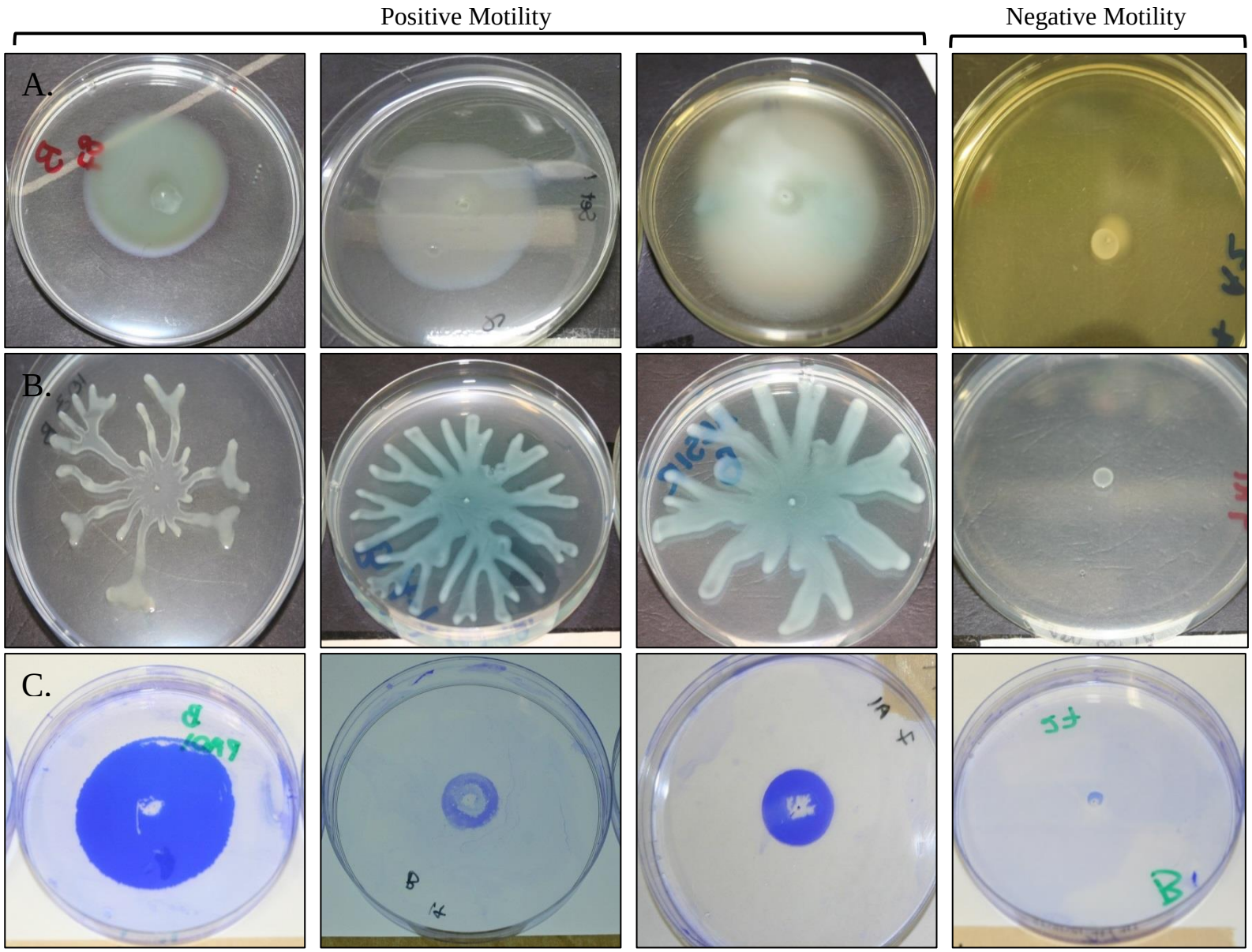
79	CF (Paediatric)	Sputum	Non-Shared CF Strain	244
80	CF (Adult)	Sputum	Non-Shared CF Strain	245
81	CF (Paediatric)	Sputum	Non-Shared CF Strain; PA14	253
82	CF (Paediatric)	Sputum	Non-Shared CF Strain	257
83	CF (Adult)	Sputum	Non-Shared CF Strain	266
84	CF (Adult)	Sputum	Non-Shared CF Strain	270
85	CF (Adult)	Sputum	Non-Shared CF Strain	274
86	CF (Adult)	Sputum	Non-Shared CF Strain	275
87	CF (Adult)	Sputum	Non-Shared CF Strain	381
88	CF (Adult)	Sputum	Non-Shared CF Strain	389
89	CF (Paediatric)	Sputum	Non-Shared CF Strain	471
90	CF (Adult)	Sputum	Non-Shared CF Strain	508
91	CF (Adult)	Sputum	Shared CF Strain; AUST-01	649
92	CF (Adult)	Sputum	Shared CF Strain; AUST-01	649
93	CF (Adult)	Sputum	Shared CF Strain; AUST-01	649
94	CF (Adult)	Sputum	Shared CF Strain; AUST-01	649
95	CF (Adult)	Sputum	Shared CF Strain; AUST-01	649
96	CF (Adult)	Sputum	Shared CF Strain; AUST-01	649
97	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
98	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
99	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
100	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
101	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
102	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
103	CF (Paediatric)	Sputum	Shared CF Strain; AUST-02	775
104	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
105	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
106	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
107	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
108	CF (Paediatric)	Sputum	Shared CF Strain; AUST-02	775
109	CF (Paediatric)	Sputum	Shared CF Strain; AUST-02	775
110	CF (Paediatric)	Sputum	Shared CF Strain; AUST-02	775
111	CF (Paediatric)	Sputum	Shared CF Strain; AUST-02	775
112	CF (Paediatric)	Sputum	Shared CF Strain; AUST-02	775
113	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
114	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
115	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
116	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
117	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
118	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
119	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
120	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
121	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
122	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
123	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
124	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775

125	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
126	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
127	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
128	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
129	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
130	CF (Paediatric)	Sputum	Shared CF Strain; AUST-02	775
131	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
132	CF (Paediatric)	Sputum	Shared CF Strain; AUST-02	775
133	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
134	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
135	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
136	CF (Paediatric)	Sputum	Shared CF Strain; AUST-02	775
137	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
138	CF (Paediatric)	Sputum	Shared CF Strain; AUST-02	775
139	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
140	CF (Paediatric)	Sputum	Shared CF Strain; AUST-02	775
141	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
142	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
143	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
144	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
145	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
146	CF (Adult)	Sputum	Shared CF Strain; AUST-02	775
147	CF (Adult)	Sputum	Non-Shared CF Strain	789
148	CF (Adult)	Sputum	Shared CF Strain; AUST-06	Novel ST*
149	CF (Paediatric)	Sputum	Shared CF Strain; AUST-06	801
150	CF (Adult)	Sputum	Shared CF Strain; AUST-06	801
151	CF (Adult)	Sputum	Shared CF Strain; AUST-06	801
152	CF (Adult)	Sputum	Shared CF Strain; AUST-06	801
153	CF (Adult)	Sputum	Shared CF Strain; AUST-06	801
154	CF (Paediatric)	Sputum	Non-Shared CF Strain	802
155	CF (Adult)	Sputum	Non-Shared CF Strain	803
156	CF (Adult)	Sputum	Non-Shared CF Strain	807
157	CF (Adult)	Sputum	Non-Shared CF Strain	808
158	CF (Adult)	Sputum	Non-Shared CF Strain	810
159	CF (Paediatric)	Sputum	Non-Shared CF Strain	821
160	CF (Paediatric)	Sputum	Non-Shared CF Strain	833
161	CF (Adult)	Sputum	Non-Shared CF Strain	845
162	CF (Adult)	Sputum	Non-Shared CF Strain	860
163	CF (Paediatric)	Sputum	Non-Shared CF Strain	870
164	CF (Adult)	Sputum	Non-Shared CF Strain	893
165	CF (Adult)	Sputum	Non-Shared CF Strain	378, 638, 797
166	CF (Adult)	Sputum	Non-Shared CF Strain	378, 638, 797
167	CF (Adult)	Sputum	Non-Shared CF Strain	378, 638, 797

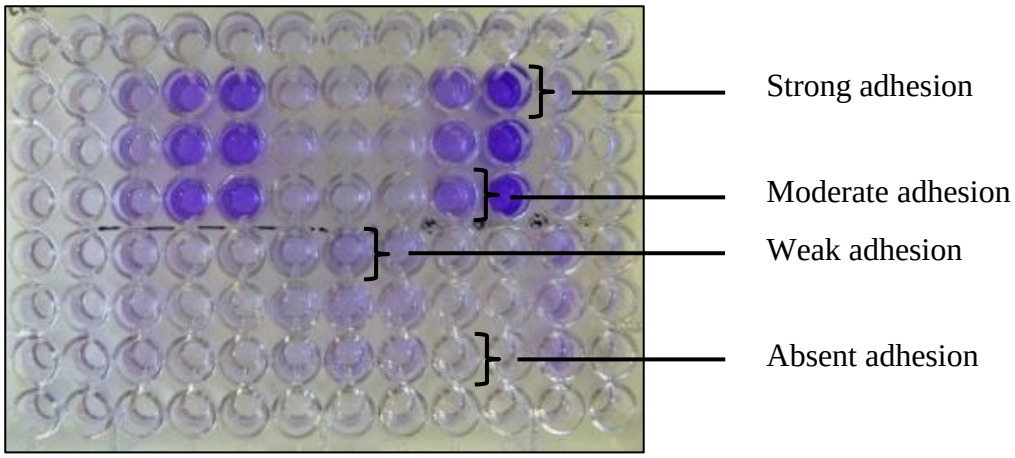
*Double-locus variant to ST-801



Appendix 3.2 Representative images of different *P. aeruginosa* colonial morphotypes, a) non-mucoid, non-pigmented, b) mucoid, non-pigmented, c) pyocyanin production, and d) pyomelanin production.



Appendix 3.3 Representative images of positive and negative *P. aeruginosa* motilities a) swim, b) swarm c) twitch (extended methodology).



Appendix 3.4 A representative image of biofilm formation of *P. aeruginosa* using the microtitre plate-based assay.

Appendix 3.5 Limits of agreements determining the reproducibility of a) swim, b) swarm and c) twitch motility assays for *P. aeruginosa* isolates tested under aerobic, microaerophilic and anaerobic conditions.

A.

	Swim								
	Aerobic			Microaerophilic			Anaerobic		
	ICC	95% CI	*P*-value	ICC	95% CI	*P*-value	ICC	95% CI	*P*-value
Environmental (n = 34)	0.842	0.657 to 0.936	<0.001	0.487	0.033 to 0.749	0.020	0.537	0.185 to 0.753	0.004
Animal (n = 14)	0.673	-0.054 to 0.928	0.030	0.922	0.799 to 0.976	< 0.001	0.774	0.437 to 0.925	0.001
Non-CF (n = 20)	0.681	0.206 to 0.894	0.007	0.286	-0.719 to 0.750	0.220	0.815	0.579 to 0.930	< 0.001
Cystic Fibrosis									
Non-Shared Strains (n = 33)	0.893	0.795 to 0.948	< 0.001	0.905	0.813 to 0.956	< 0.001	0.827	0.681 to 0.913	< 0.001
AUST-02 (n = 50)	0.987	0.979 to 0.992	< 0.001	0.944	0.910 to 0.967	< 0.001	0.659	0.457 to 0.795	< 0.001
AUST-06 (n = 6)	0.917	0.677 to 0.987	< 0.001	0.924	0.703 to 0.988	< 0.001	0.688	-0.212 to 0.952	0.045

B.

	Swarm								
	Aerobic			Microaerophilic			Anaerobic		
	ICC	95% CI	*P*-value	ICC	95% CI	*P*-value	ICC	95% CI	*P*-value
Environmental (n = 34)	0.953	0.916 to 0.975	< 0.001	0.968	0.944 to 0.983	< 0.001	0.941	0.897 to 0.969	< 0.001
Animal (n = 14)	0.999	0.966 to 0.999	< 0.001	0.945	0.868 to 0.981	< 0.001	0.987	0.970 to 0.996	< 0.001
Non-CF (n = 20)	0.697	0.355 to 0.873	0.001	0.920	0.834 to 0.966	< 0.001	0.978	0.954 to 0.991	< 0.001
Cystic Fibrosis									
Non-Shared Strains (n = 33)	0.978	0.960 to 0.988	< 0.001	0.980	0.965 to 0.990	< 0.001	0.993	0.987 to 0.996	< 0.001
AUST-02 (n = 50)	0.977	0.964 to 0.986	< 0.001	0.943	0.895 to 0.961	< 0.001	0.951	0.923 to 0.971	< 0.001
AUST-06 (n = 6)	0.996	0.985 to 0.999	< 0.001	0.885	0.551 to 0.982	0.001	0.702	-0.158 to 0.954	0.040

C.

	Twitch								
	Aerobic			Microaerophilic			Anaerobic		
	ICC	95% CI	*P*-value	ICC	95% CI	*P*-value	ICC	95% CI	*P*-value
Environmental (n = 34)	0.979	0.964 to 0.989	< 0.001	0.792	0.643 to 0.889	< 0.001	0.696	0.465 to 0.838	< 0.001
Animal (n = 14)	0.982	0.957 to 0.994	< 0.001	0.976	0.942 to 0.992	< 0.001	0.781	0.471 to 0.923	< 0.001
Non-CF (n = 20)	0.989	0.977 to 0.995	< 0.001	0.982	0.963 to 0.992	< 0.001	0.735	0.446 to 0.886	< 0.001
Cystic Fibrosis									
Non-Shared Strains (n = 33)	0.929	0.873 to 0.962	< 0.001	0.976	0.957 to 0.987	< 0.001	0.503	0.119 to 0.738	0.008
AUST-02 (n = 50)	0.884	0.816 to 0.931	< 0.001	0.854	0.767 to 0.912	< 0.001	0.495	0.193 to 0.696	0.002
AUST-06 (n = 6)	-0.437	-4.59 to 0.780	0.637	0.991	0.967 to 0.999	< 0.001	0.683	-0.233 to 0.951	0.048

Appendix 3.6 Comparison of motility and adhesion properties tested under aerobic and anaerobic atmospheric conditions for *P. aeruginosa* isolates obtained from patients with CF, non-CF human infections, animal infections, and environmental settings after 24-hours incubation.

	Swim-Positive n (%)	Swarm-Positive n (%)	Twitch-Positive n (%)	Adhesion-Positive n (%)
		Environmental Isolates (n = 34)		
Aerobic Conditions	34 (100.0)	23 (67.6)	28 (82.4)	34 (100.0)
Anaerobic Conditions	32 (94.1)	31 (91.2)	10 (29.4)	30 (88.2)
Chi-Square Value †	-	4.407	17.239	-
P value	0.493*	0.036	< 0.001	0.114 *
		Animal Isolates (n = 14)		
Aerobic Conditions	12 (85.7)	9 (64.3)	10 (71.4)	10 (71.4)
Anaerobic Conditions	9 (64.3)	5 (35.7)	4 (28.6)	10 (71.4)
Chi-Square Value †	-	1.286	3.571	-
P value	0.385*	0.257	0.059	1.000 *
		Non-CF Isolates (n = 20)		
Aerobic Conditions	19 (95.0)	13 (65.0)	12 (60.0)	17 (85.0)
Anaerobic Conditions	18 (90.0)	10 (50.0)	4 (20.0)	15 (75.0)
Chi-Square Value †	-	0.409	5.104	-
P value	1.000*	0.522	0.024	0.695 *
		All CF Isolates (n = 99)		
Aerobic Conditions	49 (49.5)	22 (22.2)	18 (18.2)	41 (41.4)
Anaerobic Conditions	37 (37.4)	16 (16.2)	3 (3.0)	18 (18.2)
Chi-Square Value †	2.699	0.814	10.441	11.685
P value	0.100	0.367	0.001	0.001

* ≥ 1 cell had expected count less than 5, therefore Fisher's Exact Test was used.

† Chi-Square value with Yates Continuity Correction.

Appendix 3.7 Comparison of two methodologies used to determine twitch motility for *P. aeruginosa* isolates †.

Conditions	Method	AUST-02 vs. Non-CF	AUST-02 vs. Animal	AUST-02 vs. Environment	AUST-02 vs. Non-Shared	AUST-02 vs. AUST-06	AUST-06 vs. Non-CF	AUST-06 vs. Animal	AUST-06 vs. Environment	AUST-06 vs. Non-Shared
O_2	2- hours	-	-	-	**< 0.001**	-	-	-	-	-
	Stain				**0.121**					
MA	24-hours	-	-	-	-	-	-	-	-	-
	Stain									
AnO_2	24-hours	-	-	-	-	-	-	**1.000 ***	**1.000 ***	-
	Stain							**0.037 ***	**0.005 ***	

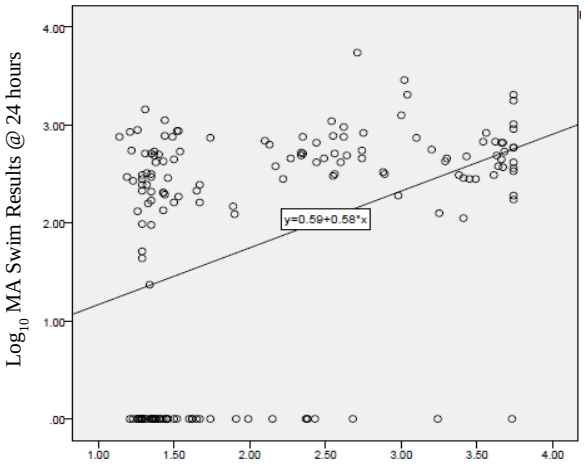
Conditions	Method	CF vs. Non-CF	CF vs. Animal	CF vs. Environment	Non-Shared vs. Non-CF	Non-Shared vs. Animal	Non-Shared vs. Environment	Animal vs. Environment	Animal vs. Non-CF	Non-CF vs. Environment
O_2	24-hours	-	-	-	-	**0.260**	-	-	-	-
	Stain					**0.022***				
MA	24-hours	-	-	-	-	-	-	**0.036 ***	-	**0.006 ***
	Stain							**0.081 ***		**0.133 ***
AnO_2	24-hours	-	**0.024 ***	-	-	**0.023 ***	**0.010**	-	-	-
	Stain		**0.240**			**0.302 ***	**0.056**			

† Chi-Square value with Yates Continuity Correction.

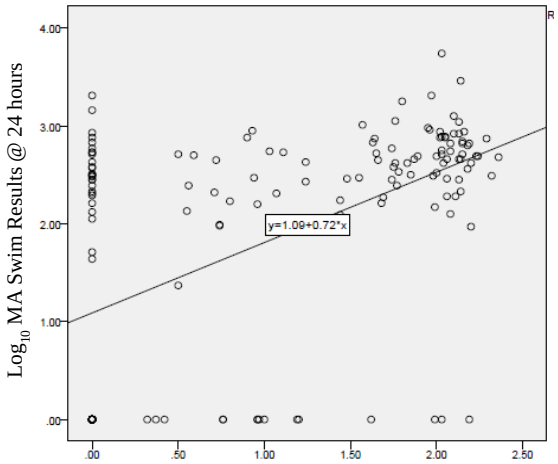
* ≥ 1 cell had expected count less than 5, therefore Fisher's Exact Test was used.

Cells containing a hyphen comprise those that showed similar results across the 24 hour and stained twitching motility assays.

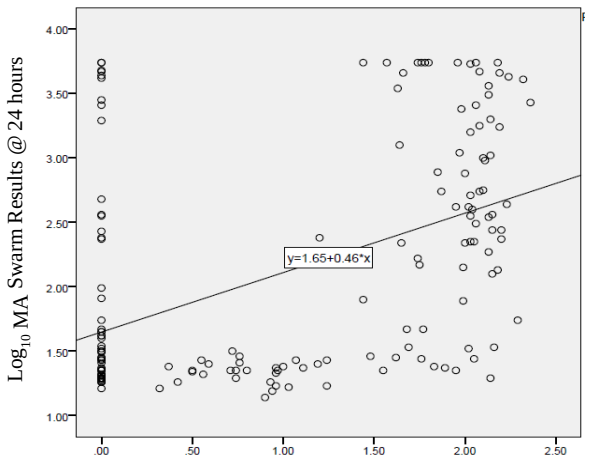
Results presented in bold represent *P*-values for the various pairwise comparisons which differed when tested in the 24 hour and stained twitching motility assays.



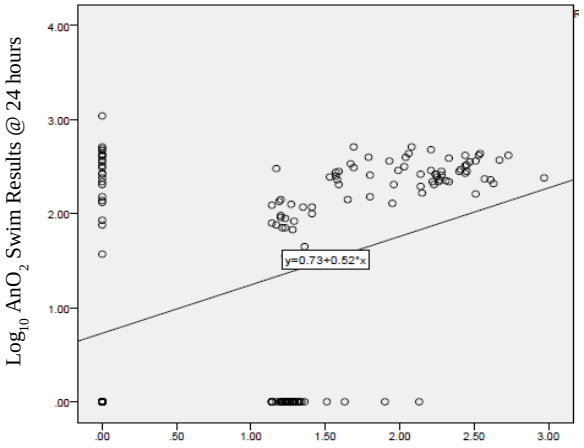
R^2 = 0.178; R = 0.422; $P \leq 0.0001$



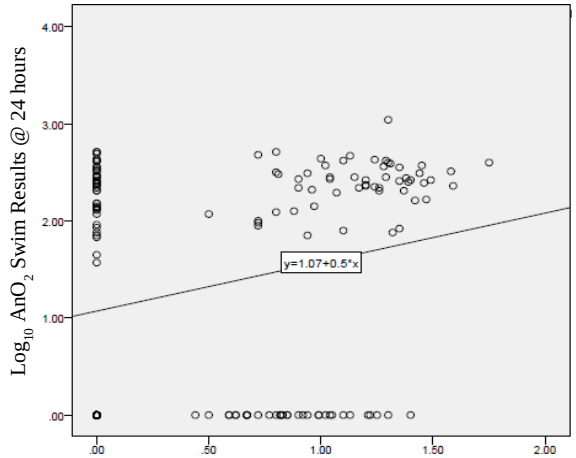
R^2 = 0.284; R = 0.533; $P \leq 0.001$



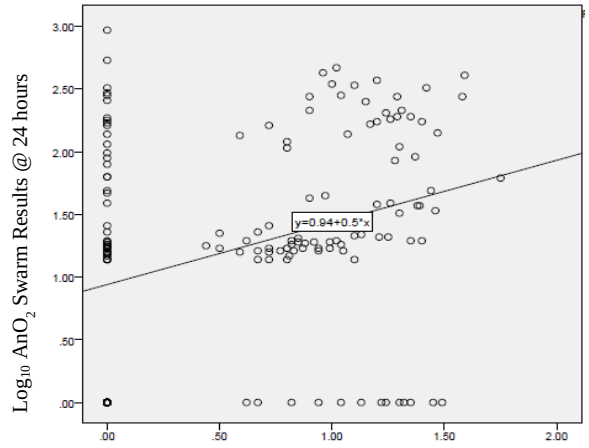
R^2 = 0.222; R = 0.471; $P \leq 0.0001$



R^2 = 0.147; R = 0.384; $P < 0.0001$



R^2 = 0.059; R = 0.243; $P = 0.002$



R^2 = 0.102; R = 0.320; $P \leq 0.001$

Appendix 3.8 Pearson correlation coefficient results of log transformed data comparing swim, swarm and twitch results following 24-hours incubation in microaerophilic and anaerobic conditions for *P. aeruginosa* isolates.

Appendix 3.9 Limits of agreements determining the reproducibility of the microtitre plate-based assay for *P. aeruginosa* isolates tested under a) aerobic, b) microaerophilic and c) anaerobic conditions.

A.

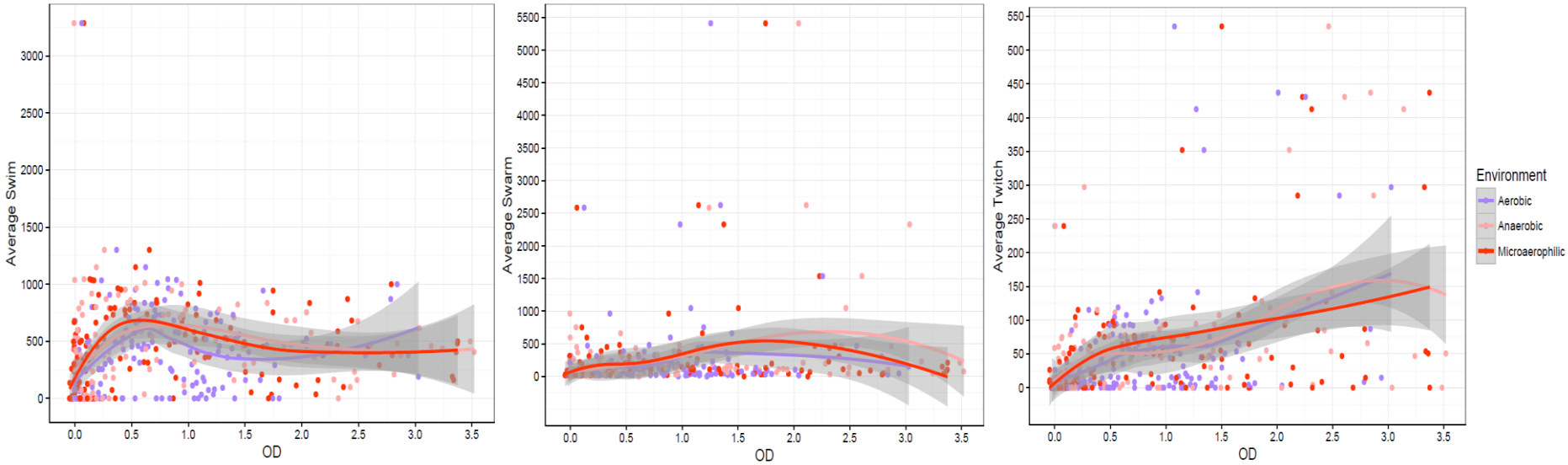
	Aerobic								
	Results from Run 1			Results from Run 2			Combined Results		
	ICC	95% CI	*P*-value	ICC	95% CI	*P*-value	ICC	95% CI	*P*-value
Environmental (n = 34)	0.965	0.939 to 0.981	<0.001	0.988	0.979 to 0.994	<0.001	0.979	0.968 to 0.986	<0.001
Animal (n = 14)	0.925	0.819 to 0.947	<0.001	0.996	0.990 to 0.999	<0.001	0.965	0.934 to 0.982	<0.001
Non-CF (n = 20)	0.953	0.902 to 0.980	<0.001	0.984	0.967 to 0.993	<0.001	0.969	0.948 to 0.983	<0.001
Cystic Fibrosis									
Non-Shared Strains (n = 33)	0.984	0.972 to 0.992	<0.001	0.944	0.898 to 0.971	<0.001	0.963	0.944 to 0.976	<0.001
AUST-02 (n = 50)	0.971	0.954 to 0.983	<0.001	0.968	0.947 to 0.981	<0.001	0.969	0.956 to 0.978	<0.001
AUST-06 (n = 6)	0.990	0.962 to 0.999	<0.001	0.935	0.748 to 0.990	<0.001	0.957	0.890 to 0.987	<0.001

B.

	Microaerophilic								
	Results from Run 1			Results from Run 2			Combined Results		
	ICC	95% CI	*P*-value	ICC	95% CI	*P*-value	ICC	95% CI	*P*-value
Environmental (n = 34)	0.961	0.932 to 0.979	<0.001	0.990	0.983 to 0.995	<0.001	0.976	0.964 to 0.984	<0.001
Animal (n = 14)	0.979	0.949 to 0.993	<0.001	0.987	0.969 to 0.995	<0.001	0.982	0.967 to 0.991	<0.001
Non-CF (n = 20)	0.989	0.978 to 0.995	<0.001	0.979	0.955 to 0.991	<0.001	0.984	0.973 to 0.991	<0.001
Cystic Fibrosis									
Non-Shared Strains (n = 33)	0.974	0.954 to 0.986	<0.001	0.994	0.988 to 0.997	<0.001	0.983	0.974 to 0.989	<0.001
AUST-02 (n = 50)	0.984	0.975 to 0.990	<0.001	0.996	0.994 to 0.998	<0.001	0.991	0.988 to 0.994	<0.001
AUST-06 (n = 6)	0.848	0.408 to 0.977	0.004	0.937	0.753 to 0.990	<0.001	0.898	0.735 to 0.968	<0.001

C.

	Anaerobic								
	Results from Run 1			Results from Run 2			Combined Results		
	ICC	95% CI	*P*-value	ICC	95% CI	*P*-value	ICC	95% CI	*P*-value
Environmental (n = 34)	0.981	0.967 to 0.990	<0.001	0.983	0.971 to 0.991	<0.001	0.982	0.973 to 0.988	<0.001
Animal (n = 14)	0.973	0.936 to 0.991	<0.001	0.963	0.910 to 0.987	<0.001	0.967	0.939 to 0.984	<0.001
Non-CF (n = 20)	0.988	0.976 to 0.995	<0.001	0.991	0.982 to 0.996	<0.001	0.989	0.982 to 0.994	<0.001
Cystic Fibrosis									
Non-Shared Strains (n = 33)	0.987	0.977 to 0.993	<0.001	0.99	0.982 to 0.995	<0.001	0.988	0.982 to 0.993	<0.001
AUST-02 (n = 50)	0.988	0.980 to 0.993	<0.001	0.945	0.911 to 0.968	<0.001	0.967	0.954 to 0.977	<0.001
AUST-06 (n = 6)	0.982	0.931 to 0.997	<0.001	0.976	0.907 to 0.996	<0.001	0.977	0.941 to 0.993	<0.001



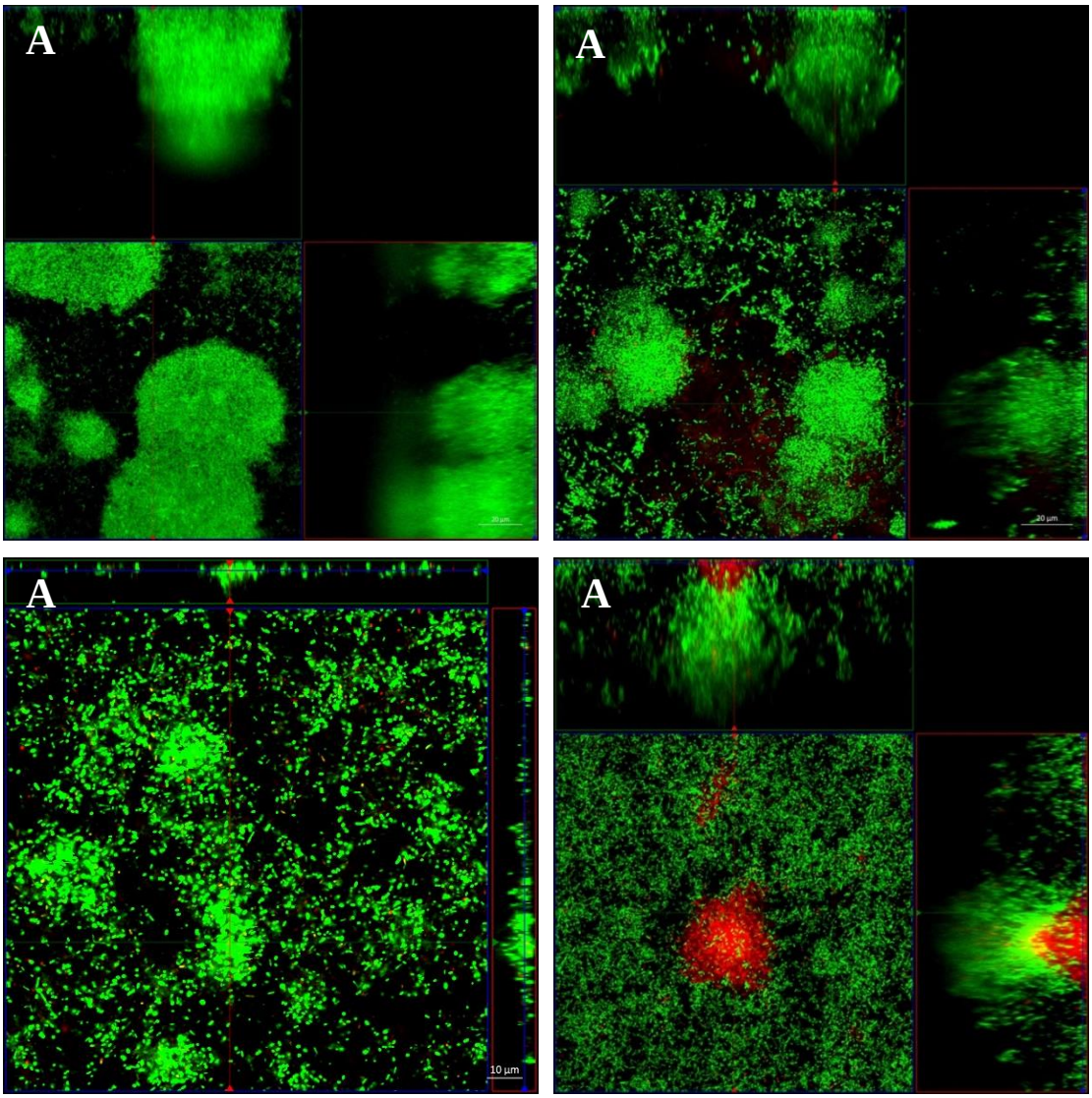
R = 0.390; $P < 0.001$ R = 0.464; $P < 0.001$ R = 0.447; $P < 0.001$

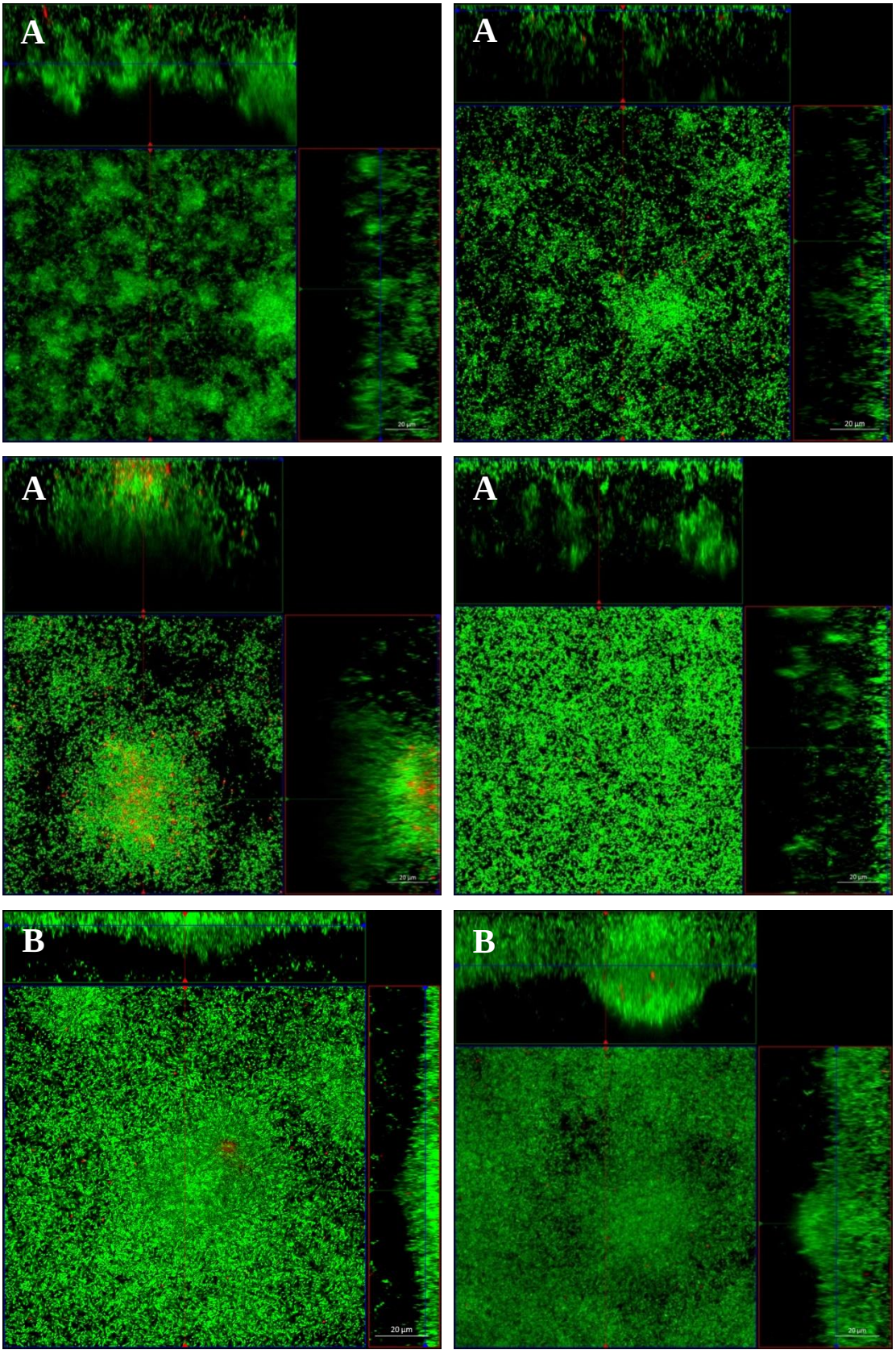
Appendix 3.10 Spearman correlation coefficient results of log transformed data comparing swim (mm^2), swarm (mm^2) and twitch (mm^2) with adhesion (OD_{595nm}) results after 24-hours incubation under each aerobic, microaerophilic and anaerobic atmospheric conditions for *P. aeruginosa* isolates.

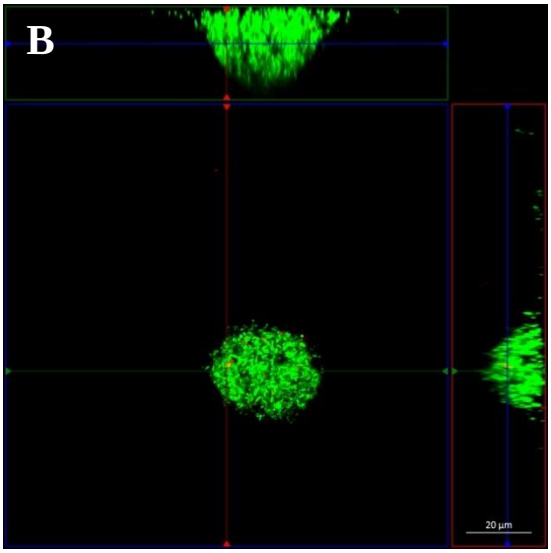
Appendix 4 Appendix to Chapter 5

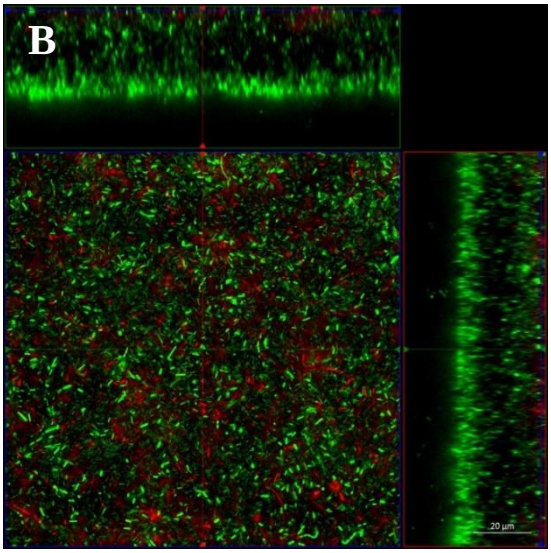
The following appendix constitutes data mentioned in Chapter 5: *Biofilm development of Pseudomonas aeruginosa*

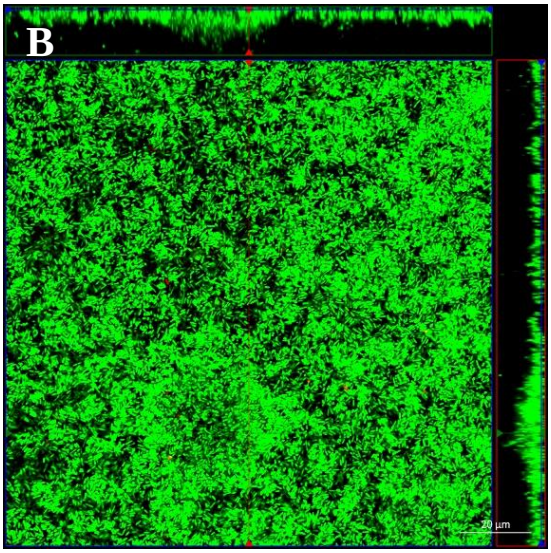
Appendix 4.1 Representative images of biofilm formation and structure from clinical (AUST-02, (A)), environmental (B) and reference strain (C) of *Pseudomonas aeruginosa.*

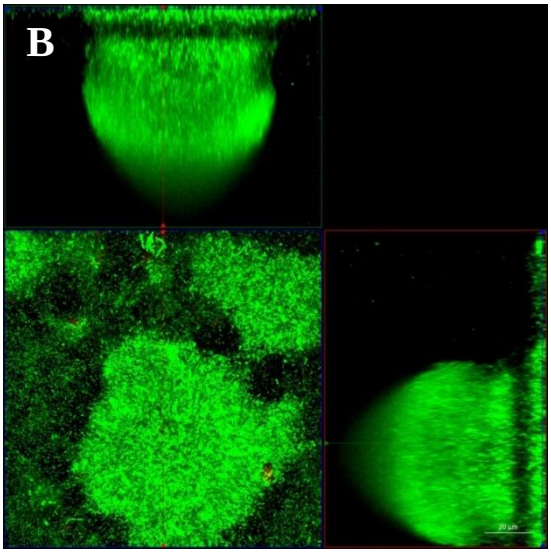


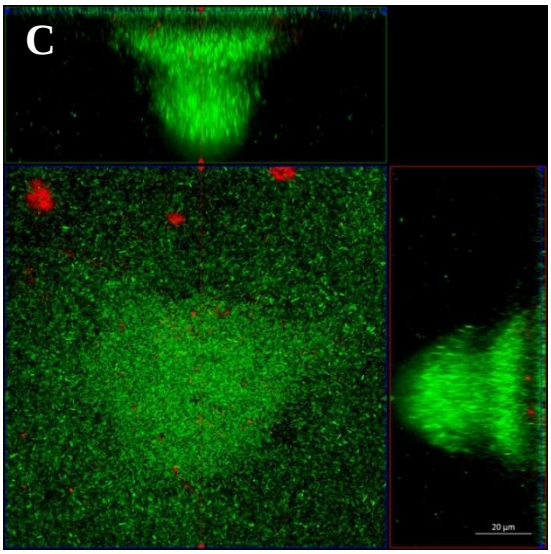

A
A
A
A
B
B


B
20 µm


B
20 µm


B
20 µm


B


C
20 µm

Appendix 5 Appendix to Chapter 6

The following appendix constitutes data mentioned in Chapter 6: *Genomic analysis of Pseudomonas aeruginosa*

Appendix 5.1 The five most significant SNPs and indels identified from GWAS analysis for defined phenotypic characteristics of *P. aeruginosa* isolates.

Appendix 5.2 List of References for Appendix 1

Appendix 5.1 The five most significant SNPs and indels identified from GWAS analysis for defined phenotypic characteristics of *P. aeruginosa* isolates.

Morphology	SNP		*P*-value	Function	Reference	INDEL		*P*-value	Function	Reference
Swim O_2	PA3535	Ala687Thr	1.5×10^{-6}	Putative serine protease	[1]	*aprE*	upstream modifier	4.4×10^{-7}	Alkaline protease secretion protein. This gene was upregulated when isolates were swarming compared to swimming	[2]
	PA3536	Ser122Gly	1.5×10^{-6}	Hypothetical protein		PA1536	Ser19FS	4.4×10^{-7}	Hypothetical protein	
	algE	Arg10Trp	1.5×10^{-6}	Outer membrane protein associated with alginate synthesis and export	[3-5]	*pscK*	Cys152del	4.4×10^{-7}	Under swarm conditions this gene is up-regulated, whereas in a swarm mutant this gene is down-regulated	[2, 6]
	algE	Gly79Ala	1.5×10^{-6}	Outer membrane protein associated with alginate synthesis and export	[3-5]	PA1874	Ser150_Gly151dup	4.4×10^{-7}	Novel efflux pump shown to enhance resistance of cells within a biofilm structure compared to planktonic cells	[7]
	PA3641	Ala239Gly	1.5×10^{-6}	Putative amino acid permease	[8]	PA2049	Pro16_Leu17dup	4.4×10^{-7}	Hypothetical protein	
Swim MA	PA3535	Ala687Thr	1.1×10^{-6}	Putative serine protease	[1]	*pchC*	upstream modifier	2.8×10^{-7}	Pyochelin biosynthetic protein required for production and associated with iron uptake	[9, 10]
	PA3536	Ser122Gly	1.1×10^{-6}	Hypothetical protein		*aprE*	upstream modifier	5.2×10^{-7}	Alkaline protease secretion protein. This gene was upregulated when isolates were swarming compared to swimming	[2]
	algE	Arg10Trp	1.1×10^{-6}	Outer membrane protein associated with alginate synthesis and export	[3-5]	PA1536	Ser19FS	5.2×10^{-7}	Hypothetical protein	
	algE	Gly79Ala	1.1×10^{-6}	Outer membrane protein associated with alginate synthesis and export	[3-5]	*pscK*	Cys152del	5.2×10^{-7}	Under swarming conditions this gene is up-regulated, whereas in a swarm mutant this gene is down-regulated	[2, 6]
	PA3641	Ala239Gly	1.1×10^{-6}	Putative amino acid permease	[8]	PA1874	Ser150_Gly151dup	5.2×10^{-7}	Novel efflux pump shown to enhance resistance of cells within a biofilm structure compared to planktonic cells	[7]
Swim AnO_2	Not included due to population bias					Not included due to population bias				
Swarm O_2	PA5021	Pro317Gln	0.01	Loss of this gene has been associated with a decrease in the production of pyocyanin in a plant model	[11]	No significant indels				
	PA1613	Gln679Glu	1.7×10^{-2}	Outer membrane protein associated with early *P. aeruginosa* airway infections	[12]					
	PA0625	Val220Ala	1.8×10^{-2}	Evidence of upregulation in conditions of oxidative stress such as anaerobic conditions or exposure to hydrogen peroxide	[13, 14]					
	flgK	Asn372Ser	2.2×10^{-2}	Flagella hook associated protein. Mutations in *flgK* render the cells non-motile (swim) and impair attachment to abiotic surfaces	[15-19]					
Swarm MA	PA1908	Gly91Ala	2.5×10^{-10}	Probable major facilitator superfamily transporter		PA3298	Lys67_Met94del	1.9×10^{-7}	Hypothetical protein	
	PA4881	Ile52Val	1.5×10^{-8}	Hypothetical protein		PA0636	Gly655del	1.5×10^{-6}	Hypothetical protein	
	clpB	Val846Leu	2.6×10^{-8}	Chaperone protein, mutants display reduced swimming and have less flagella present than wild types	[20]	PA2927	Ala55_Thr80del	1.5×10^{-6}	Hypothetical protein	
	PA0495	Ala97Val	3.7×10^{-8}	Hypothetical protein		*fimV*	Gly682_Asp683insGlyAspLeuGlySer	7.3×10^{-6}	Mutations within the *fimV* gene results in the loss of twitching motility	[21, 22]
	PA1907	Gly162Asp	3.7×10^{-8}	Hypothetical protein		*opmQ*	Gln34FS	3.7×10^{-5}	Outer membrane protein comprising the efflux system PvdRT-OpmQ which has been associated with the secretion of pyoverdin	[23-25]
Swarm AnO_2	PA1997	Gly215Asp	2.0×10^{-8}	Probable AMP binding enzyme has been associated with increased drug susceptibility	[26]	*xcpQ*	Glu417_Ser418delinsAla	5.0×10^{-6}	Outer membrane secretin associated with Type II Secretion System and the transport of proteins across the outer membrane, no defect to swarming of biofilm development in *xcpQ* mutants	[27-30]
	hpaA	Ile477Val	2.0×10^{-8}	Transcriptional regulator which functions as a metabolic enzyme, has been associated with increased resistance to aminoglycosides	[31]	PA4156	Gly471FS	6.6×10^{-6}	*fvbA* gene which enable *P. aeruginosa* utilise siderophores from *Vibrio sp.* as an iron source	[32, 33]
	PA0209	Ala229Val	5.0×10^{-8}	Up-regulated during chemotaxis displaying increased expression of twitching	[34]	PA2268	Pro241FS	1.0×10^{-4}	Hypothetical protein	
	purN	Glu73Gln	1.2×10^{-6}	Phosphoribosylaminoimidazole synthetase		*fimV*	Gly682_Asp683insGlyAspLeuGlySer	2.0×10^{-4}	Mutations within the *fimV* gene results in the loss of twitching motility	[21, 22]
	PA3025	Pro389Ala	1.2×10^{-6}	Probable FAD-dependent glycerol-3-phosphate dehydrogenase		PA2793	Leu16_Cys17dup	3.0×10^{-4}	Hypothetical protein	
Twitch O_2	PA4781	Leu158Phe	1.6×10^{-8}	Cyclic-Di-GMP. Mutations in this gene have been associated with reduced swarming and twitching motility and biofilm development	[35, 36]	PA2583	Gly13FS	2.4×10^{-6}	Probable sensor and or response regulator hybrid associated with increased resistance to Colistin	[37]
	PA4782	Ala6Val	1.6×10^{-8}	Hypothetical protein		PA4156	Gly471FS	2.9×10^{-6}	*fvbA* gene which enable *P. aeruginosa* utilise siderophores from *Vibrio sp.* as an iron source	[32, 33]
	PA5484	Glu54Lys	1.6×10^{-8}	Mutation within *kinB* reduces biofilm production and prevents swimming motility	[38, 39]	*xcpQ*	Glu417_Ser418delinsAla	7.1×10^{-6}	Outer membrane secretin associated with Type II Secretion System and the transport of proteins across the outer membrane, no defect to swarming of biofilm development in *xcpQ* mutants	[27-30]
	PA5492	Pro189Leu	1.6×10^{-8}	Hypothetical protein		*pilQ*	Gln628FS	8.0×10^{-6}	Mutations within the *pilQ* gene results in the loss of twitching motility and surface fimbriae	[40-43]
	PA0683	Val191Ile	1.6×10^{-8}	HxcY, homologue of *xcpY*, part of the hxc gene cluster which may be involved in the type II secretion system	[30]	PA3340	Glu601dup	1.5×10^{-5}	Hypothetical protein	

Appendix 5.1, continued The five most significant SNPs and indels identified from GWAS analysis for defined phenotypic characteristics of *P. aeruginosa* isolates.

Morphology	SNP		*P*-value	Function	Reference	INDEL		*P*-value	Function	Reference
Twitch MA	No SNP's identified due to population bias					*xcpQ*	Gl417_Ser418delinsAla	1.5×10^{-7}	Outer membrane secretin associated with Type II Secretion System and the transport of proteins across the outer membrane, no defect to swarming of biofilm development in *xcpQ* mutants	[27-30]
						fimV	Gly682_Asp683insGlyAspLeuGlySer	9.0×10^{-7}	Mutations within the *fimV* gene results in the loss of twitching motility	[21, 22]
						PA4156	Gly471FS	3.0×10^{-6}	*fvbA* gene which enable *P. aeruginosa* utilise siderophores from Vibrio sp. As an iron source	[32]
						treA	Ser34_Trp35insSerSer	5.0×10^{-6}	Periplasmic trehalase precursor associated with the metabolism of trehalose, therefore cannot utilise trehalose as a carbon source	[44]
						mpl	Met38FS	6.0×10^{-6}	Ligase involved with the biosynthesis of bacterial cell wall peptidoglycan	[45, 46]
Twitch AnO_2	No significant correlation								No significant correlation	
Adhesion O_2	PA2069	Ala480Glu	7.0×10^{-7}	Probable carbamoyl transferase, not known to be involved in biofilm development	[47]	PA3340	Glu601dup	1.5×10^{-5}	Hypothetical protein	
	PA3292	Ser202Pro	7.0×10^{-7}	Hypothetical protein		*opr86*	Arg668FS	2.6×10^{-5}	*opr86* is essential for survival. Antibodies against this gene have been shown to inhibit biofilm formation	[48]
	gcvP1	Ser98Pro	7.0×10^{-7}	Glycine cleavage system protein		*opr86*	Thr673_Met675del	2.6×10^{-5}	*opr86* is essential for survival. Antibodies against this gene have been shown to inhibit biofilm formation	[48]
	PA0017	Lys195Gly	2.2×10^{-5}	Hypothetical protein		PA1401	Val130del	6.3×10^{-5}	Hypothetical protein	
	PA0669	Ser885Thr	2.2×10^{-5}	Probable DNA polymerase alpha chain	[49, 50]	PA4156	Gly471FS	6.6×10^{-5}	*fvbA* gene which enable *P. aeruginosa* utilise siderophores from Vibrio sp. As an iron source	[32, 33]
Adhesion MA	PA0711	Leu49Phe	8.0×10^{-7}	Hypothetical protein, which has been shown to display high levels of recombination	[51]	PA4156	Gly471FS	1.4×10^{-6}	*fvbA* gene which enable *P. aeruginosa* utilise siderophores from Vibrio sp. As an iron source	[32, 33]
	PA2291	Cys24Gly	8.0×10^{-7}	Probable glucose-sensitive porin, found in lower levels in AUST-01 compared to reference strains	[52]	*opr86*	Arg668FS	4.0×10^{-6}	*opr86* is essential for survival. Antibodies against this gene have been shown to inhibit biofilm formation	[48]
	PA0690	Asn1364Asp	2.5×10^{-6}	Phosphate depletion regulated TPS partner A, PdtA		*opr86*	Thr673_Met675del	7.7×10^{-6}	*opr86* is essential for survival. Antibodies against this gene have been shown to inhibit biofilm formation	[48]
	trpA	Ile148Leu	9.0×10^{-6}	Tryptophan synthase alpha chain		*xcpQ*	Glu417_Ser418delinsAla	7.0×10^{-5}	Outer membrane secretin associated with Type II Secretion System and the transport of proteins across the outer membrane, no defect to swarming of biofilm development in *xcpQ* mutants	[27-30]
	PA0144	Val191Ile	9.0×10^{-6}	Hypothetical protein which has been shown to be activated during quorum sensing	[53]	PA3340	Glu601dup	1.0×10^{-4}	Hypothetical protein	
Adhesion AnO_2	PA2125	Lys67Thr	1.8×10^{-6}	Associated with the cupA gene cluster which encodes the fimbrial structure enabling bacterial adhesion to abiotic surfaces under anaerobic conditions	[54]	PA0636	Gly566del	1.5×10^{-6}	Hypothetical protein	
	PA2283	Ala114Val	1.8×10^{-6}	Hypothetical protein		PA1797	Ser256_Ala257dup	9.0×10^{-5}	Putative beta-lactamase	[55]
	PA3080	Ser18Leu	1.8×10^{-6}	Hypothetical protein		PA2268	Pro241FS	9.8×10^{-5}	Hypothetical protein	
	PA3360	Ala14Thr	1.8×10^{-6}	Probable secretion protein		*pilQ*	Gln628FS	1.0×10^{-4}	Mutations within the *pilQ* gene results in the loss of twitching motility and surface fimbriae	[40-42]
	PA1997	Gly215Asp	1.9×10^{-6}	Probable AMP binding enzyme has been associated with increased drug susceptibility	[26]	PA3298	Lys67_Met94del	2.0×10^{-4}	Hypothetical protein	

Appendix 5.2 List of References for Appendix 5.1

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